Genetic Variations and Phylogenetic Relationships of Tribe Forsythieae (Oleaceae) Based on RAPD Analysis

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ABSTRACT

RAPD analysis was performed to discuss the taxonomic status and phylogenetic relationships among the tribe Forsythieae and related groups. Two hundred and eighteen scorable polymorphic bands were detected from fourteen oligonucleotide primers. From the results of RAPD analysis by Nei and Li's genetic distance, each individuals *of Abeliophyllum distichum* showed high genetic relationships with ranging from 0.085 to 0.301, also the genus *Forsythia* showed from 0.042 to 0.655 among the species and populations. But, *Abeliophyllum* and *Forsythia* showed distinct dissimilarity, ranging from 0.610 to 1.258. And genetic differences among the population of *Forsythia* were 0.042 in *F. koreana*, 0.275 in *F. saxatilis*, 0.275 in *F. ovata*, 0.279 in *F. nakaii*, and 0.249 in *F. viridissima*. The UPGMA phenogram of tribe Forsythieae based on the results of RAPD analysis were presented that *Abeliophyllum* is distinct genus different from *Forsythia*. NJ tree which applied as the outgroups *Fontanesia* and *Jasminum* was derived, and it showed that tribe Forsythieae might be a monophyletic group. The genus *Fontanesia* was showed as sister group of tribe Forsythieae. Among the populations of taxa in *Forsythia*, *F. koreana* and *F. saxatilis* were more closely related, and *F. ovata* and *F. nakaii* were very closely related to *F. japonica*. And *Fontanesia* was the sister group of tribe Forsythieae.

Key words : NJ, Oleaceae, RAPD, relationship, tribe Forsythieae, UPGMA

INTRODUCTION

The tribe Forsythieae H. Taylor ex L.A.S. Johnson, belong to the subfamily Jasmonoideae under the family Oleaceae Hoffmanns. & Link, is distributed in Northern Hemisphere, especially in the temperate regions of Far Eastern Asia - Korea, China and Japan(Mechior, 1964). The tribe Forsythieae is consisted of the genus *Abeliophyllum* Nakai and *Forsythia* Vahl, and it is that all taxa are reported endemic plants of eastern Asia except *F. europaea* Degen & Bald which is distributed in South-East Europe (Willis, 1973; Mabberley, 1987).

The genus *Abeliophyllum* Nakai is composed of only *A. distichum*, Korean endemic plant even though several intra-specific taxa had been recorded as varieties and forms (Nakai, 1922; Lee, T.B. 1976). When Nakai (1919b) recorded *A. distichum* firstly, and he suggested that *Abeliophyllum* is closely related to *Fontanesia* Labill. by fan-shape samara. Thereafter, Nakai (1920) reported that *Abeliophyllum* might be closer with

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Forsythia Vahl based on the raceme, corolla-tube and terraced pith. Therefore, floral structure of *Abeliophyllum* is similar to that of *Forsythia*, also the shape and structure of fruits resemble those of *Fontanesia*.

The genus *Forsythia* consists of about 12 taxa in the world (Rehder, 1940; Willis, 1973; Bailey and Bailey, 1976). *Forsythia* is reported as a monophyletic group in Oleaceae (Kim, 1999; Kim *et al.*, 2000), and is differ from related genus by the morphological characters such as fruit shape (capsule), pith of stem (hollow) and flower color (yellow). Since Palibin (1900) had reported *F. viridissima* Lindl. about the Korean *Forsythia*, and Nakai (1917, 1919a, 1942) reported four species of *F. koreana, F. saxatilis* and *F. ovata* as the new species. Lee(1976) recorded *F. nakaii* T. Lee according to the treatment by Uyeki (1940), and Lee(1984) described *F. saxatilis* var. *laceolata* S. Lee and *F. saxatilis* var. pilosa S. Lee.

There have been many controversals on the systematic relationship and phylogenetic status among *Abeliophyllum*, *Forsythia* (tribe Forsythieae) and *Fontanesia* (tribe Fontanesiae). The representatives are as follows : 1) *Abeliophyllum* is closer to *Fontanesia* than *Forsythia* by the morphology and anatomy of fruits (Taylor, 1945; Johnson, 1957) and by the aperture type of pollen (Lee and Park, 1982b). 2) *Abeliophyllum* is closer to *Forsythia* than *Fontanesia* by the basic chromosome numbers (x=14: Taylor, 1945; Maekawa, 1962; Lim and Ko, 1989) and by the shape of petals and the pith type of stem (Lee and Park, 1982a). 3) there were no definite results on systematic relationships among them and phylogenetic status of *Abeliophyllum* by flavonoids patterns (Harborne and Green, 1980) and chemotaxonomic immunnological study of proteins (Piechura and Firbrothers, 1983). 4) *Abeliophyllum* should be treated with a species of *Forsythia* based on the overall taxonomic tendency which emphasize the floral structure in Oleaceae (P.S. Green, personal comm.). Above stated opinions, therefore, three related genera showed very close relationships in geneology and systematics as mentioned above.

Recently, several molecular systematic studies have been performed on nrDNA ITS sequences and chloroplast DNA (Kim, 1999; Kim *et al.*, 2000; Wallander and Albert, 2000; Kim *et al.*, 2004). Those studies were proposed that *Abeliophyllum* is the sister group of *Forsythia* and might be included in tribe Forsythieae.

Genetic study has been supported the traditional research by external morphology, and it was get at the root of phylogenetic system and evolutionary process base on characters of a special gene (Doyle *et al.*, 1992). New technique developed to generate markers used to establish genetic linkage maps uses the polymerase chain reaction (PCR) to produce random amplified polymorphic DNA (RAPD) makers (Williams *et al.*, 1990). RAPD markers are generated using short DNA primers. These makers are easily generated, can be rapidly analyzed, and use small amounts of DNA. RAPD analysis for useful genetic makers can help determine the relationship, variation, and differentiation within and between species and population (Adams and Demeke, 1993; Lynch and Milligan, 1994; Heibel *et al.*, 1999).

In this study, we tried to investigate the genetic variation of the tribe Forsythieae and to suggest the clues on assessing the phylogentic status of *Abeliophyllum*. And we discussed the systematic relationships and evaluated the phylogenetic status of tribe Forsythieae based on the RAPD analysis.

MATERIALS AND METHOD

Materials

Experimental plants were collected in the field and some taxa were taken from the leaves of dried specimens in herbaria. Examined individuals were

Table 1. Materials and collection data of tribe Forsythieae and Fontanesa, Jasminum which were used in RAPDs analysis

Taxa	Localities	Symb.
tribe Forsythieae		
Abeliophyllum distichum Nakai	Naibyeonsan, Jeollabuk-do (Apr. 4, 2000) Kim & Jeon 0044	AD1
	Maecheon-ri, Chungcheongbuk-do (Apr. 5, 2000) Kim & Jeon 0027	AD2
	Songdeok-ri, Chungcheongbuk-do (Apr. 6, 2000) Kim & Jeon 0010	AD3
Forsythia koreana Nakai	Mt. Gyeryong, Chungcheongnam-do (Apr. 17, 2000) Kim & Jeon 0144	FK1
	Yongjeong-ri, Chungcheongbuk-do (Apr. 7. 2000) Kim & Jeon 0001	FK2
Forsythia saxatilis Nakai	Imsil-gun, Jeollabuk-do (Apr. 4, 2000) Kim & Jeon 0048	FS1
	Hongreung Arboretum, Origin Mt. Bukhan (Apr. 9, 2000) Kim & Jeon 0022	FS2
Forsythia ovata Nakai	RBGKew 000-73.21135 (Apr. 2, 1999)	FO1
	Mt. Seolak, Gangwon-do (Apr. 21, 2000) Kim & Jeon 0100	FO2
Forsythia nakaii T. Lee	Gwangreung Arboretum, Origin Mt. Jangsu (Apr. 9, 2000) Kim & Jeon 0154	FN1
	Hongreung Arboretum, Origin Mt. Jangsu (Apr. 21, 2000) Kim & Jeon 0080	FN2
Forsythia japonica Makino	Hongreung Arboretum, (Apr. 2, 1999)	FJA
Forsythia suspensa (Thunb.) Vahl	RBGKew 264-79, 06640 WHHP (Apr. 2, 1999)	FSU
Forsythia viridissima Lindl	Euiseong-gun, Gyeongsangbuk-do (Apr. 5, 2000) Kim & Jeon 0033	FV1
	RBGKew 422-16, 42214 AARB, China (Apr. 2, 1999)	FV2
Frosythia europaea Degen & Baldacci	RBGKew 367-32, 36906 TASH Yugoslavia (Apr. 2, 1999)	FEU
Forsythia giraldiana Ling	RBGKew 1995-1652 SICH 1137 China (Apr. 2, 1999)	FGI
Forsythia × intermedia	RBGKew 000-73. 21134 (Apr. 2, 1999)	FIN
tribe Fontanesieae		
Fontanesia fortunei Carr.	Cheolipo Arboretum, Chungcheongnam-do(Apr. 5, 2000)	FFO
Fontanesia phyllyreoides Labill	Gwangreung Arboretum, (May 21, 2001) Kim & Jeon 0156	FPH
tribe Jasminoideae		
Jasminum nudiflorum LindleyKim & Jeon 0018	Gwanak Arboretum, (Apr. 9, 2000)	JNU

prepared into voucher specimens, which were deposited in the TUT at Daejeon University.

The plant materials used in this study and their collection localities are given in Table 1. Leaves used as sources of DNA were collected from natural populations. All plant materials were kept in vinyl zipper bag with silica-gel until returned to the laboratory and stored at -70℃ in the laboratory until use.

Methods

(1) DNA extraction - Total genomic DNA was extracted from fresh leaf pulverized in liquid nitrogen using the 2X CTAB buffer (Doyle and Doyle, 1987). DNA sample were stored at -20℃ until use.

(2) RAPD - Randomly amplified polymorphic DNA in each genomic DNA was amplified by 35 cycles with No. 1-100 oligo-primers by NAPS (Univ. of British Columbia). Amplifications were performed in 25㎕ reactions containing 10-50ng DNA, 200 M dNTP (equimolar), 0.5 units AmpliTaq DNA polymerase (Perkin & Elmer, Cetus), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, and primers at 1.0 M. Before the PCR cycles, PCR mixture was pre-denatured at 94℃ for 2 minutes. The PCR cycle consisted of 1 minute at 94℃ for denaturation, 2 minutes at 44℃ for annealing, and 2 minutes at 72℃ for extension. After 35 cycles, the PCR reactions were incubated at 72℃ for 7 minutes to complete final extension. After the useful primers were screened, we conducted three times repetitive reactions with same primers to confirm their availability. Agarose gel electrophoresis were employed to check the amplified DNA products by 1.3% agarose gel with 1×10-4% EtBr. And the correct band positions were determined with 1D image analysis software (Kodak, 2001, ver. 3.5) and were confirmed by naked eye again.

(3) Data Analysis - All RAPD band assigned a number and treated as a unit character coded as 0 (absent) or 1 (present) by the operational taxonomic units (OTUs), and the data matrix was conducted. The genetic variations were calculated by Nei and Li's genetic distance (1979) with the distance program of PAUP (Swofford, 2003, ver. 4.08b). The UPGMA phenogram were produced by the RAPD results, and the Nei and Li's distance was to use to construct a Neighbor-Joining tree (NJ tree, Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Thirty primers were screened from one hundred primers which were used in this study. Most of reacted 30 primers showed the relatively high G+C contents (60-80%). This results supported the previous investigation on RAPD analysis (William *et al.*, 1990; Fritsch *et al.*, 1993). Fourteen primers among them presented the same results from all the taxa after three times repeat reaction (Table 2). The considered RAPD markers which showed consistent amplification were found in the ranges from 200 bp to 2,300 bp, and ten to twenty bands per primers were amplified according to the taxa. RAPD analyses showed that genetic differences among the taxa. Also, RAPD bands showed almost same patterns among the populations within the species (Fig. 1-3). The two hundred and twenty six scorable band makers from 14 primers were applied to generate the genetic dissimilarity matrix by Nei and Li's distance (Table 3), also UPGMA phenogram was produced from the data matrix (Fig. 4). Also we performed the NJ tree analysis to discuss the phylogenetic relationships among the treated taxa with several outgroups (Fig. 5).

Each individuals of *Abeliophyllum distichum* showed higher genetic relationship with ranging from 0.085 to 0.301 (Table 3). This result supported the previous RAPD data which intraspecific taxa of *Abeliophyllum distichum* might be synonymized to mother species (Kim *et al.*, 2002). Among the populations of *Forsythia*

Table 2. The code and sequences of primer analysed, total number of bands and fragment size which were used in this study

Primer	Sequence (5'-3')	Total no. of bands	Fragment size range(bp)
NAPS-01	CCT GGG CTT C	17	200 - 2100
NAPS-02	CCT GGG CTT G	16	200 - 2200
NAPS-04	CCT GGG CTG G	14	300 - 2000
NAPS-05	CCT GGG TTC C	13	300 - 1900
NAPS-06	CCT GGG CCT A	12	350 - 2100
NAPS-20	TCC GGG TTT G	18	350 - 2000
NAPS-39	TTA ACC GGG C	13	250 - 2300
NAPS-40	TTA CCT GGG C	16	300 - 2100
NAPS-42	TTA ACC GGG C	18	400 - 2000
NAPS-43	AAA ACC GGG G	17	300 - 2100
NAPS-75	GAG GTC CAG A	17	350 - 2000
NAPS-78	GAG CAC TAG C	19	300 - 2100
NAPS-82	GGG CCC GAG G	8	400 - 1900
NAPS-84	GGG CGC GAG T	20	300 - 2000
Total		218	
Mean/primer		15.5	

showed the close genetic relationships from 0.042 to 0.655. And it showed genetic differences 0.042 in *F. koreana*, 0.275 in *F. saxatilis*, 0.275 in *F. ovata*, 0.279 in *F. nakaii*, and 0.249 in *F. viridissima*. But, RAPD results could not support the distinct resolution to assess the modernized taxonomic system among the infrageneric species of *Forsythia*. It showed the similar results with the precious studies from numeric analysis (Lee, 1984) and from chloroplast DNA RFLP analysis (Kim, 1999). Therefore, further study should be needed to discuss the phylogenetic relationships and establish the taxonomic system of *Forsythia* even though those results were related from the sexual structure (heterostyly), frequent hybrid mechanism, different distributional patterns, and etc. Also, there were obvious genetic differences between *Abeliophyllum* and *Forsythia*, ranging from 0.610 to 1.258. This result supported that *Abeliophyllum* is the independent genus which comprise only in Korea (Nakai, 1919b; Paik, 1994).

Based on the UPGMA and NJ tree by RAPD analysis, tribe Forsythieae were clustered independently showing monophyly (Fig. 4, 5). This result supported the close relationship between *Abeliophyllum* and *Forsythia* (Taylor, 1945; Maekawa, 1962; Lee and Park, 1982a; Lim and Ko, 1989). Also current study which *Fontanesia* (tribe Fontanesiae) were clustered as sister group of tribe Forsythieae supported the nrDNA ITS data (Kim *et al.*, 2000; Kim *et al.*, 2004) and chloroplast DNA studies (Kim, 1999; Wallander and Albert, 2000; Kim *et al.*, 2004). And two groups were well defined with very close relationships among the species of *Forsythia* (*koreana-saxatilis*, *ovata-nakaii-japonica*). This result was partly different from the chloroplast DNA RFLP analysis (Kim, 1999) and numerical analysis (Lee, 1984). Also,

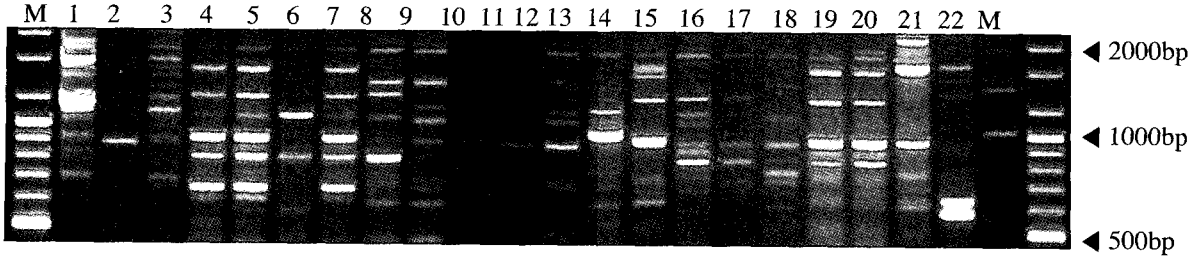

Fig. 1. A RAPD profile generated by primer NAPS-42.
M : Molecular Maker
Lane 1-3 : *Abeliophyllum distichum* Nakai
Lane 4-5 : *Forsythia koreana* Nakai
Lane 6-8 : *Forsythia saxatilis* Nakai
Lane 9-10 : *Forsythia ovata* Nakai
Lane 11-12 : *Forsythia nakaii* T. Lee
Lane 13 : *Forsythia japonica* Makino
Lane 14 : *Forsythia suspensa* Vahl
Lane 15-16 : *Forsythia viridissima* Lindl
Lane 17 : *Forsythia europaea*
Lane 18 : *Forsythia giraldiana*
Lane 19 : *Forsythia* × *intermedia*
Lane 20 : *Fontanesia fortunei*
Lane 21 : *Fontanesia phyllyreoides* Labill
Lane 22 : *Jasminum nudiflorum*

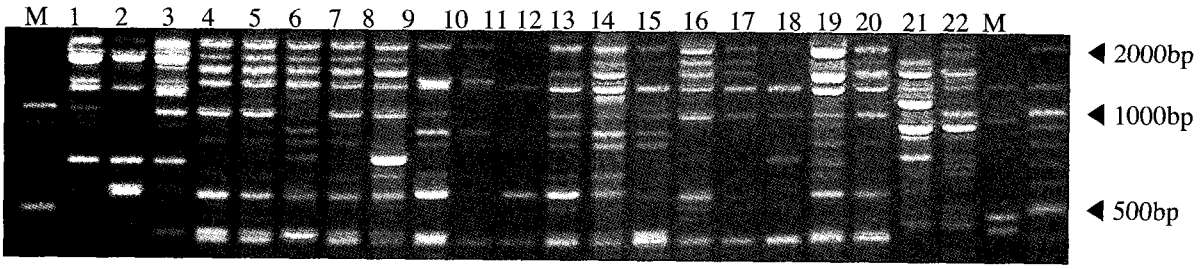

Fig. 2. A RAPD profile generated by primer NAPS-43.
The numbers and letters designating the lanes are same in Fig. 1.

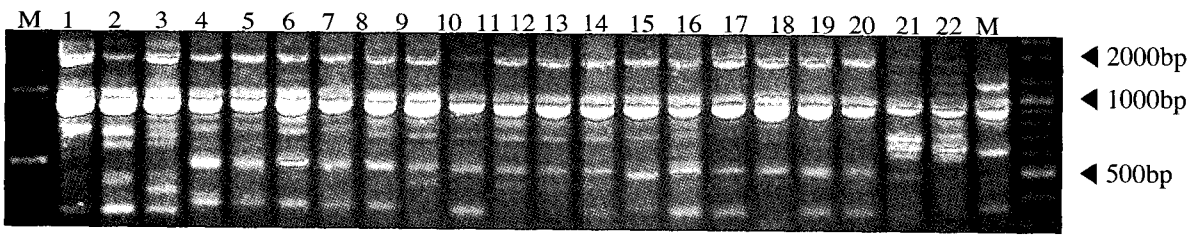

Fig. 3. A RAPD profile generated by primer NAPS-82.
The numbers and letters designating the lanes are same in Fig. 1.

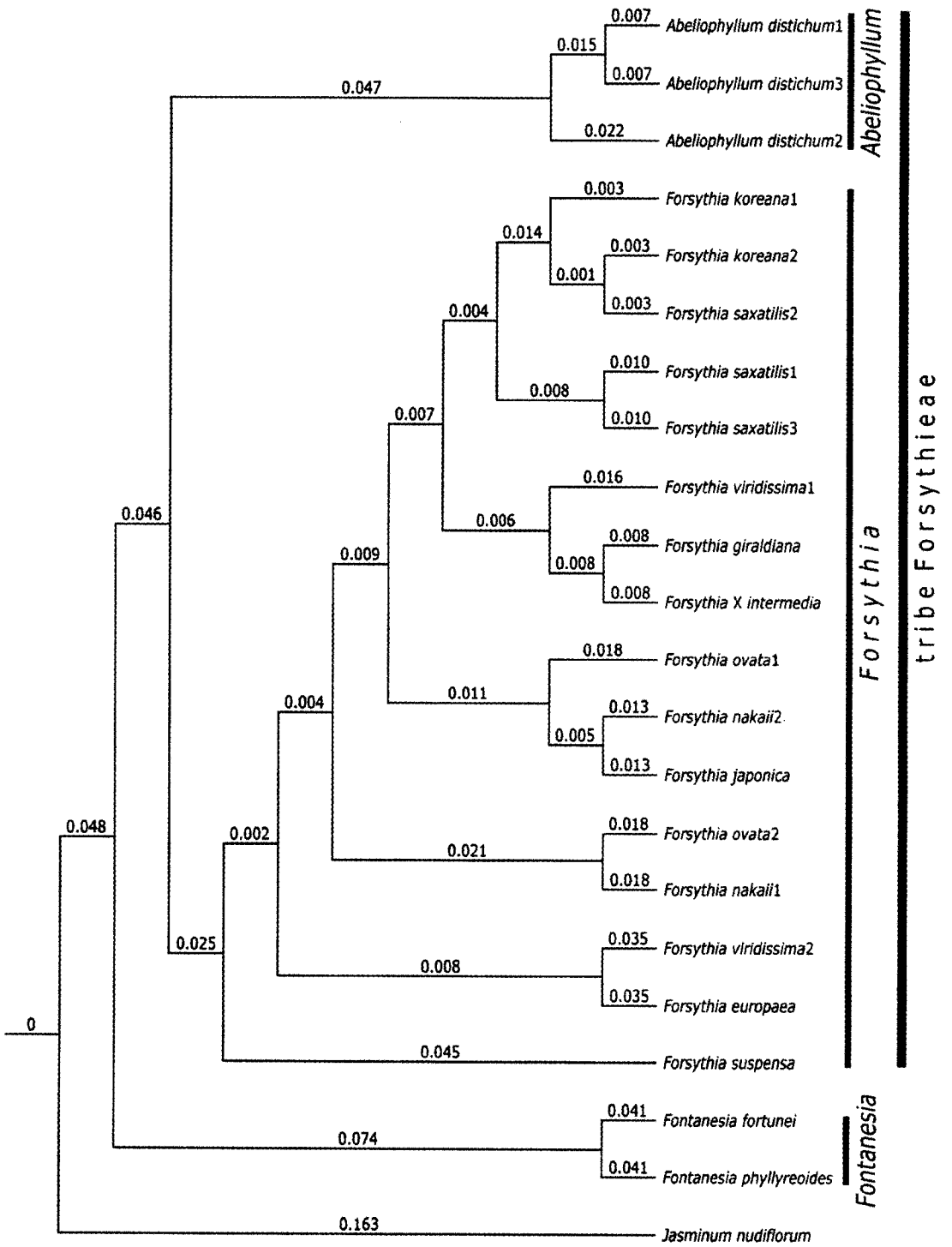

Fig. 4. The UPGMA phenogram of tribe Forsythieae based on the results of RAPD analysis.

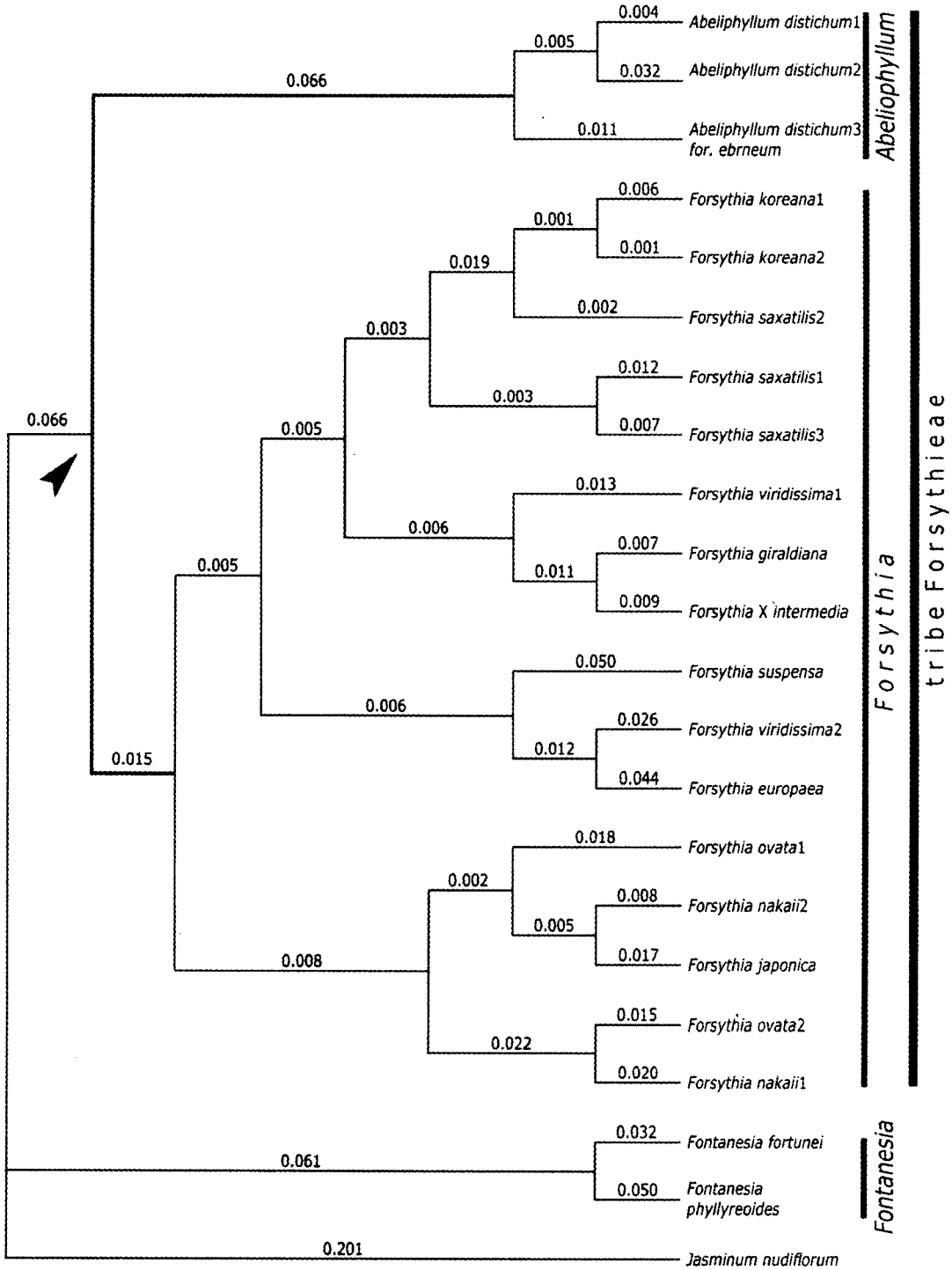

Fig. 5. The Neighbor-Joining tree of tribe Forsythieae based on the results of RAPD analysis.

F. europaea which is disjunct from majority of its NE Asian taxa showed very close relationship not with *F. giraldiana* but with *F. viridissima* and *F. suspensa*. And this study was concrete with flavonoid compound distribution (Harborne and Green, 1980) and cpDNA RFLP analysis (Kim, 1999) which *F. intermedia* was not hybrid of *F. suspensa* and *F. viridissima*.

In conclusion, RAPD analysis was very useful and applicable to assess genetic variations and the systematic relationships in tribe Forsythieae and related group. Also, we will try to establish modernized taxonomic system of genus *Forsythia* and related taxa and to elucidate the phylogenetic relationship with further more studies.

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