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Full Length Research Paper

Phylogeny of Korean Hornbeam (*Carpinus turczaninovii*) based on nuclear ribosomal ITS sequence

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The genus *Carpinus* belonging to Coryloideae, Betulaceae, has significant economic and ornamental importance. This study was undertaken with the aim to understand the genetic diversity among eighteen isolates of *Carpinus turczaninovii* collected from different geographical regions of Korea, using ribosomal RNA (rRNA) internal transcribed spacer (ITS) sequences, to compare the infraspecific-phylogenetic relationships among *C. turczaninovii* in Korea, and some known *Carpinus* plants. The size variation of sequenced rRNA ITS regions was not seen, with 215, 162, 222 bp of ITS1 region, 5.8S rRNA gene, ITS2 region, respectively. However, some certain nucleotide variations resulted in genetic diversity. In the genus *Carpinus*, *C. turczaninovii* closely genetic with *Castanopsis kawakamii*, *Carpinus orientalis*, *Carpinus monbeigiana*, and *Calyptranthes polynenra* formed one monophyletic clade, while *Carpinus betulus* and *Carpinus laxiflora,* respectively formed one monophyletic clade. In the present ITS sequence analysis, *Carpinus fangiana*, *Carpinus coreana*, *Carpinus japonica*, and *Carpinus caroliniana* were considered as Out Group, compared to *C. turczaninovii* group. The highest intraspecific variation was found between *C. caroliniana* and *C. japonica*. The least intraspecific variation was obtained between *C. turczaninovii* and *C. coreana*. The results will help further understanding *Carpinus* infraspecies population and their phylogenetic analysis.

Key words: *Carpinus*, Hornbeam, *Carpinus turczaninovii*, molecular identification, nuclear ribosomal RNA, internal transcribed spacer, infraspecific-phylogenetic relationship.

INTRODUCTION

Coryloideae is one of the two subfamilies of Betulaceae, consisting of four genera: ironwoods (*Carpinus* L.), hazelnuts (*Corylus* L.), hop hornbeams (*Ostrya* Scopoli), and the Chinese endemic shrub, *Ostryopsis* Decne (Prantl, 1894; Winkler, 1904; Mabberley, 1997). The other subfamily Betuloideae is composed of two genera: birches (*Betula* L.) and alders (*Alnus* Mill.). Most species of six genera are ecologically and economically important (Abbe, 1974; Furlow, 1990). Their phylogenetic relationships have been strongly supported by recent studies on the basis of *rbc*L, internal transcribed spacer (ITS), morphology, and various combined date sets (Bousquet et al., 1992; Savard et al., 1993; Chen et al., 1999; Whitcher and Wen, 2001; Yoo and Wen, 2002). And there is no controversy about the division of the Coryloideae into two subfamilies.

However, some taxonomists are inclined to rank subfamily Betulaceae either at the tribal (Prantl, 1894; Winkler, 1904) or the familial level (Hutchinson, 1926; Dahlgren, 1983). Kuprianova (1963) emphasized the importance of some characters such as pollen apertures, and suggested that *Carpinus*, *Ostrya* and *Ostryopsis* formed a third family Carpinaceae, leaving only *Corylus*

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in the Corylaceae. Abbe (1974) agreed that *Carpinus* and *Ostrya* could be separated from the Coryloideae, to form a third tribe, Carpineae according to floral morphology. Brunner and Fairbrothers (1979) supported a broadly defined family of Betulaceae based on serological evidence. Takhtajan (1997) recognized the division of Corylaceae into two subfamilies Carpinoideae (*Carpinus*, *Ostrya*, and *Ostryopsis*) and Coryloideae (*Corylus*).

Coryloideae consists of four genera, comprising 120 ~ 150 species, of which *Carpinus* is the largest genus of Coryloideae with ~ 35 extant species (Rehder, 1960; Heywood, 1993; Chen, 1994). The genus has an inter-continental disjunct distribution in Europe (*Carpinus betulus* and *Carpinus orientalis*), North America (*Carpinus caroliniana* and *Carpinus tropicalis*), and eastern Asia (ca. 30 species; Chen, 1994). Although, most of the species are restricted to far eastern Asia, including China, Taiwan, Korea and Japan, *Carpius* is more widely scattered.

Several phylogenetic studies were conducted to construct the infrageneric relationships, including those based on morphology (Winkler, 1904; Hu, 1964; Li and Cheng, 1979), morphology and *rbc*L (Bousquet et al., 1992), *mat*K sequences (Kato et al., 1998), ITS sequences (Sun et al., 2010a, b) and ITS combined with morphology (Whitcher and Wen, 2001; Yoo and Wen, 2002). About the infrageneric relationship within the genus *Carpinus*, many botanists brought forward their own attitudes (Winkler, 1904; Hu, 1933).

Winkler (1904) monographied the genus and provided an infrageneric classification, recognizing two sections (section *Distegocarpus* and *Eucarpinus*) primarily based on morphology of bracts. Hu (1933) recognized two subgenera (*Distegocarpus* and *Carpinus*) and established six new series (example, *Fangiana* and *Cordatae* for subgenus *Distegocarpus* and *Betulae*, *Monbeigianae*, *Pubescentes*, and *Polyneurea* for subgenus *Carpinus*) based on morphology of fruiting bracts, nutlets, and inflorescences.

Li and Cheng (1979) further recognized *Carpinus* into three subsections with the section *Carpinus* (*Carpinus*, *Monbeigianae*, and *Polyneurae*) (Li and Cheng, 1979). Thus, to understand assured infrageneric classification within the genus *Carpinus*, more stable and advanced classification methods were needed to investigate, and more samples were needed to be fixed within a phylogenetic framework.

Besides these, infraspecies genetic diversity has also received much attention in recent year using different approaches and many distinct taxa (Antonovics, 1968; Hamrick et al., 1992; Silander and Antonovics, 1979; Sun et al., 2010a, b). Because the divergence among populations may occur as a result of microevolutionary changes in isolated populations in different environments that produce individuals with different ecological tolerances to physical factors, resulting in the differentiation of divergence ecotypes (Jung et al., 2004). The actual geographical distribution of a species may be reflective of these changes over geological time. *Carpinus turczaninovii* Hance [*Carpinus turzcaninovii* Hance was firstly established in J. Linn. Soc. Bot. 10:203 (1869)], also named Korean Hornbeam, is one of the main species of the genus *Carpinus*, especially in Korea. Until recently, no detailed information on the distribution of the genetic variation has been found in this species, *C. turczaninovii*.

In this study, we have constructed the infrageneric phylogeny for different *C. turczaninovii* populations of Korea based on the nuclear ribosomal ITS sequence data, and framed the position of *C. turczaninovii* within a phylogenetic framework of *Carpinus* with analysis of some known related species in *Carpinus*.

MATERIALS AND METHODS

Plant materials

18 different *C. turczaninovii* isolates were collected from various areas of South Korea. Fresh leaf tissues were harvested and sampled within liquid nitrogen. Until DNAs were isolated, the fresh leaf tissues were always stored in -80°C. The voucher data for all populations, abbreviations and GenBank accession numbers are summarized in Table 1. The known ITS sequences of *Carpinus* plant species are listed in Table 2, with their accession numbers and references.

PCR amplification of the nuclear ribosomal ITS sequences

Total genomic DNAs were extracted using the CTAB method of Doyle and Doyle (1987). DNA amplifications were performed in 20 µl reactions containing ca. 10 ng genomic DNA, 20 nM Tris buffer (pH 8.3) with 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20, and 0.15% mM of each dNTP, 1 µM of each primer, 1 units of Taq DNA polymerase (Promega, USA). The ITS regions were amplified using common ITS primer sets ITS5, 5'-GAAAGTAAAAGT-CGTAACAAGG-3' and ITS4, 5'- TCCTCCGCTTATTGATATGC-3' (White et al., 1990). Double-stranded PCR products were produced via following program: 35 cycles of denaturation at 95˚C for 1 min, annealing at 53˚C for 1 min, and a final extension step at 72°C for 1.5 min, a 7 min final extension cycle at 72°C followed the 35 cycles to ensure the completion of novel strands. All PCR products were purified before DNA sequence analysis using a QIAquick PCR Purification Kit (QIAGEN, Korea) according to the manufacturer's instructions. Purified PCR products were then sequenced at Advancing through Genomics MACROGEN Service (Korea).

Sequence analysis

Analogue was detected with BLAST on the server on NCBI (http://www.nncbi.nih.gov). The sequences of eighteen isolates were analyzed using DNAMAN 6.0 software according to the methods of Sun et al. (2010a, b). Jaccard coefficients used to represent identity among the ecotypes were calculated by similarity coefficient [Sj = a/(a+u)]. In the total ITS region, ITS1 and ITS2 region, '1' was used for base variation and '0' was used for no variation; 'a' represents the number of the same bases and 'u' represents the number of different bases between the two ecotypes.

Table 1. Accession number and geographical origin of eighteen isolates of *Carpinus turczaninovii* in Korea.

S/N	Isolate	Accession number	Geographical origin
1	SCGD	JF831016	Samcheok-Si Geundeok-Myeon
2	GJNB1	JF831017	Geoje-Si Nambu-Myeon
3	GJNB2	JF831018	Geoje-Si Nambu-Myeon
4	GJNB3	JF831019	Geoje-Si Nambu-Myeon
5	BABS1	JF831020	Buan-Gun Byeonsan-Myeon
6	BABS2	JF831021	Buan-Gun Byeonsan-Myeon
7	TASW1	JF831022	Taean-Gun Sowon-Myeon
8	TASW2	JF831023	Taean-Gun Sowon-Myeon
9	TASW3	JF831024	Taean-Gun Sowon-Myeon
10	GHPY	JF831025	Goheung-Gun Pungyang-Myeon
11	HNSS1	JF831026	Haenam-Gun Samsan-Myeon
12	HNSS2	JF831027	Haenam-Gun Samsan-Myeon
13	GHGS1	JF831028	Ganghwa-Gun Gilsang-Myeon
14	GHGS2	JF831029	Ganghwa-Gun Gilsang-Myeon
15	OJYH1	JF831030	Ongjin-Gun Yeongheung-Myeon
16	OJYH2	JF831031	Ongjin-Gun Yeongheung-Myeon
17	OJYH3	JF831032	Ongjin-Gun Yeongheung-Myeon
18	JSHA	JF831033	Jeongseon-Gun Hwaam-Myeon

Table 2. ITS sequences of twenty-five known *Carpinus* plant species with their accession numbers and references.

S/N	Species	Accession number	References
1	*Carpinus fangiana*	AJ783633	Forest et al. (2005)
2	*Carpinus betulus*	AF465186	Selosse et al. (2004)
3	*Carpinus japonica*	AJ783635	Forest et al. (2005)
4	*Carpinus japonica*	AJ783637	Forest et al. (2005)
5	*Carpinus betulus*	AF297362	Erdogan and Mehlenbacher (2002)
6	*Carpinus sp.* Wen 9187	FJ011729	Yoo and Wen (2008)
7	*Carpinus turczaninowii*	AF432056	Yoo and Wen (2001)
8	*Carpinus coreana*	AF432033	Yoo and Wen (2001)
9	*Carpinus sp.* Tibet 1942	FJ011732	Yoo and Wen (2008)
10	*Carpinus betulus*	AJ783636	Forest et al. (2005)
11	*Carpinus turczaninovii*	FJ011734	Yoo and Wen (2008)
12	*Carpinus sp.* Wen 9031	FJ011731	Yoo and Wen (2008)
13	*Carpinus orientalis*	FJ011725	Yoo and Wen (2008)
14	*Carpinus orientalis*	FJ011724	Yoo and Wen (2008)
15	*Carpinus orientalis*	AF432049	Yoo and Wen (2001)
16	*Carpinus betulus* UASWS0410	HM235960	Lefort et al. (2010)
17	*Carpinus turczaninowii*	AF081518	Chen et al. (2002)
18	*Carpinus polyneura*	FJ011726	Yoo and Wen (2008)
19	*Carpinus kawakamii*	FJ011720	Yoo and Wen (2008)
20	*Carpinus monbeigiana*	AF432047	Yoo and Wen (2001)
21	*Carpinus caroliniana*	AF783634	Forest et al. (2005)

RESULTS

Total ITS sequence analysis of *C. turczaninovii*

The total rRNA ITS sequences were amplified with common primer set, ITS5 and ITS4 from eighteen isolates of *C. turczaninovii* in Korea, containing complete ITS1 region, 5.8S rRNA gene and ITS2 region sequences. These sequences have been referred to NCBI GenBank database, with accession numbers from JF831016 to

Table 3. Nucleotide variation sites in the ITS1 and ITS2 regions among eighteen isolates of *Carpinus turczaninovii*.

Isolate	Nucleotide variation site								
	ITS 1 region (bp)					ITS 2 region (bp)			
	2	28	45	59	76	207	463	501	512
BABS1	c	c	c	t	g	t	g	c	t
BABS2	c	c	c	c	c	t	g	c	t
GHGS1	c	c	c	c	c	t	g	c	t
GHGS2	c	c	c	c	c	g	g	c	t
GHPY	c	c	c	t	g	t	g	c	t
GJNB1	c	c	c	c	c	t	g	c	t
GJNB2	c	c	c	c	c	t	g	c	t
GJNB3	c	c	c	c	c	t	g	t	t
HNSS1	c	c	c	c	c	t	g	c	t
HNSS2	c	c	c	c	c	t	g	c	t
JSHA	c	c	t	c	c	t	g	c	t
OJYH1	c	c	c	c	c	t	g	c	t
OJYH2	c	c	c	c	c	t	g	c	a
OJYH3	c	c	c	c	c	t	g	c	t
SCGD	c	c	c	c	c	t	g	c	t
TASW1	t	c	c	c	c	t	g	c	t
TASW2	c	c	c	c	c	t	g	c	t
TASW3	c	t	c	c	c	t	a	t	t

JF831033 (Table 1).

The eighteen total ITS sequences nearly did not show size variation, all including 599 bp. There was also no size variation existing in the ITS1 region, 5.8S rRNA gene, ITS2 region sequences. Due to infraspecific phylogeny analysis, G+C contents of eighteen total ITS sequences showed very narrow, but determinate variations, ranging from 59.43% (TASW3) to 60.10% (GHGS2). The G+C contents of ITS sequences of other isolates were mainly at either value, 59.93 or 59.77%, which were collectively caused by the ITS1 region sequence variation and the ITS2 region sequence variation. The nucleotide variation was located at night nucleotide sites, of which five sites belonged to ITS1 region, while four sites belonged to ITS2 region (Table 3). The G+C contents of the ITS1 region sequence ranged from 60.47% to 60.93%, mostly being 60.93% among these sequences, while those of the ITS2 region sequence ranged from 62.16 to 63.06%, mostly being 62.61%.

Among all ITS1 region sequences of eighteen isolates, both BABS1 and GHPY had one more nucleotide G accession and two nucleotide C default, while JSHA and TASW3 showed only one nucleotide C default, resulting in decreased G+C contents (60.47%). There was no nucleotide variation appearing in the intervening part, 5.8S rRNA gene sequence among eighteen isolate in this study. And the G+C contents of 5.8S rRNA gene sequence were all 54.94% among eighteen isolates used in this study. Among the ITS2 region sequences of eighteen isolates, OJYH2, though, showed one nucleotide variation at 512 bp, with T substituted by A, its G+C content was not influenced by this change; TASW3 had one nucleotide G default, while GJNB3 had one nucleotide C default, both resulting in slightly decreased G+C contents (62.16%); GHGS2 showed one more nucleotide G accession, inducing increased G+C content (63.06%). Integrated with all G+C contents, TASW3, in particular, showed the lowest G+C content in ITS1 (60.47%), ITS2 (62.16%) and consequentially total ITS region sequences (59.43%).

Symmetric matrix of Jaccard coefficients of total ITS region sequences showed some identity ranging from 95.6 to 100% (Table 4). The least Jaccard coefficient appeared between JSHA and GHGS1, while the largest Jaccard coefficients appeared between GHPY and BABS1, and SCGD and GJNB1. Some samples were collected from the same geographical region, like BABS1 and BABS2, GHGS1 and GHGS2, GJNB1, GJNB2 and GJNB3, HNSS1 and HNSS2, OJYH1, OJYH2 and OJYH3, and TASW1, TASW2 and TASW3. However, there were still genetic variation existing in the ITS region sequences, showing 1.2 ~ 0.2% genetic diversity.

According to the result of phylogenetic tree, all sequences amplified in this experiment were divided into two clades, with 98% identity between them (Figure 1). Among them, GHGS2, HNSS2 and JSHA formed one clade, the other isolates formed another clade, composed by BABS1 and GHPY group, BABS2, GJNB2, TASW2, GJNB3 and GHGS1 group, GJNB1, SCGD, OJYH1, and

Table 4. Symmetric matrix of Jaccard coefficients (% identity) in total ITS regions between eighteen isolates of *Carpinus turczaninovii*.

Isolate	BABS1	BABS2	GHGS1	GHGS2	GHPY	GJNB1	GJNB2	GJNB3	HNSS1	HNSS2	JSHA	OJYH1	OJYH2	OJYH3	SCGD	TASW1	TASW2	TASW3
BABS1	100																	
BABS2	99.1	100																
GHGS1	98.8	99.7	100															
GHGS2	98.7	99.0	98.9	100														
GHPY	100	99.1	98.8	98.7	100													
GJNB1	99.1	99.4	99.2	99.0	99.1	100												
GJNB2	99.1	100	99.9	99.0	99.1	99.4	100											
GJNB3	99.5	99.8	99.8	99.7	99.5	99.8	99.8	100										
HNSS1	99.0	98.9	98.6	99.0	99.0	99.0	99.0	99.8	100									
HNSS2	99.0	99.7	99.7	99.8	99.0	99.7	99.7	99.8	99.7	100								
JSHA	97.0	95.9	95.6	99.2	97.0	96.2	96.0	99.7	95.9	99.7	100							
OJYH1	99.1	98.8	99.3	99.0	99.1	99.9	99.6	99.8	98.7	99.7	96.2	100						
OJYH2	98.0	98.8	98.5	98.6	98.0	98.8	98.8	99.5	98.7	99.0	97.2	98.8	100					
OJYH3	99.1	99.3	99.0	99.0	99.1	99.6	99.3	99.8	99.2	99.7	96.0	99.4	98.8	100				
SCGD	99.1	99.4	99.0	99.0	99.1	100	99.4	99.8	97.1	99.7	96.2	99.7	98.8	99.2	100			
TASW1	99.0	99.3	99.0	98.9	99.0	99.6	99.3	99.7	98.6	99.5	96	99.7	98.7	99.3	99.3	100		
TASW2	99.1	99.9	99.6	99.0	99.1	99.4	99.9	99.8	98.7	99.7	95.9	99.4	98.8	99.3	99.3	99.2	100	
TASW3	99.0	99.6	99.3	98.6	99.0	99.5	99.6	99.7	99.6	99.4	98.9	99.6	98.5	99.6	99.5	99.4	99.6	100

TASW1 group, HNSS1 and TASW3 group, OJYH3, and OJYH2. Each isolates in the same group showed nearly 100% similarity to each other, and each group had 99% identity with other either group.

Total ITS sequence analysis of the genus *Carpinus*

In this study, molecular classifications based on ITS sequence were investigated to understand further inter-specific relationships among twenty-five known *Carpinus* species. Among them, *C. betulus* and *C. orientalis* were species from Europe and *C. caroliniana* was species from North American, while other species were all distributed in Eastern Asia (Chen, 1994). According to the intergeneric phylogenetic tree, *C. fangiana* showed high identity with *Carpinus japonica* and *Carpinus coreana* Nakai (99%), and these three species with *C. caroliniana* were considered to be Out Group, relative to *C. turczaninovii* clade (Figure 2).

The *C. turczaninovii* clade was composed by *C. turczaninovii* group (Group 1), *C. betulus* (Group 2) and *Carpinus laxiflora* (Group 3). Among them, *C. turczaninovii* as well as *C. coreana*, *C. orientalis*, and *C. turczaninovii* formed one subgroup (Subgroup 1), with being sister to the other subgroup composed by *C. kawakamii*, *C. monbeigiana*, and *C. polyneura* (Subgroup 2). Our results of different isolates of *C. turczaninovii* in Korea were very similar to the known sequences of *C. turczaninovii*, with the identity of nearly 100% (Figure 2). However, the infraspecific variation was also detected in *C. turczaninowii* and *C. japonica* for which these samples had geographical divergence. Despite *C. betulus* and *C. orientalis* were distributed in Europe naturally, both species showed relatively high similarity to *C. turczaninovii* in the ITS region sequence.

DISCUSSION

Carpinus, the hornbeam genus, established by Linnaeus (1737), is an ideal model to construct the biogeographical history of the Northern Hemisphere. In this study, we investigated the infraspecific relationships among different isolates

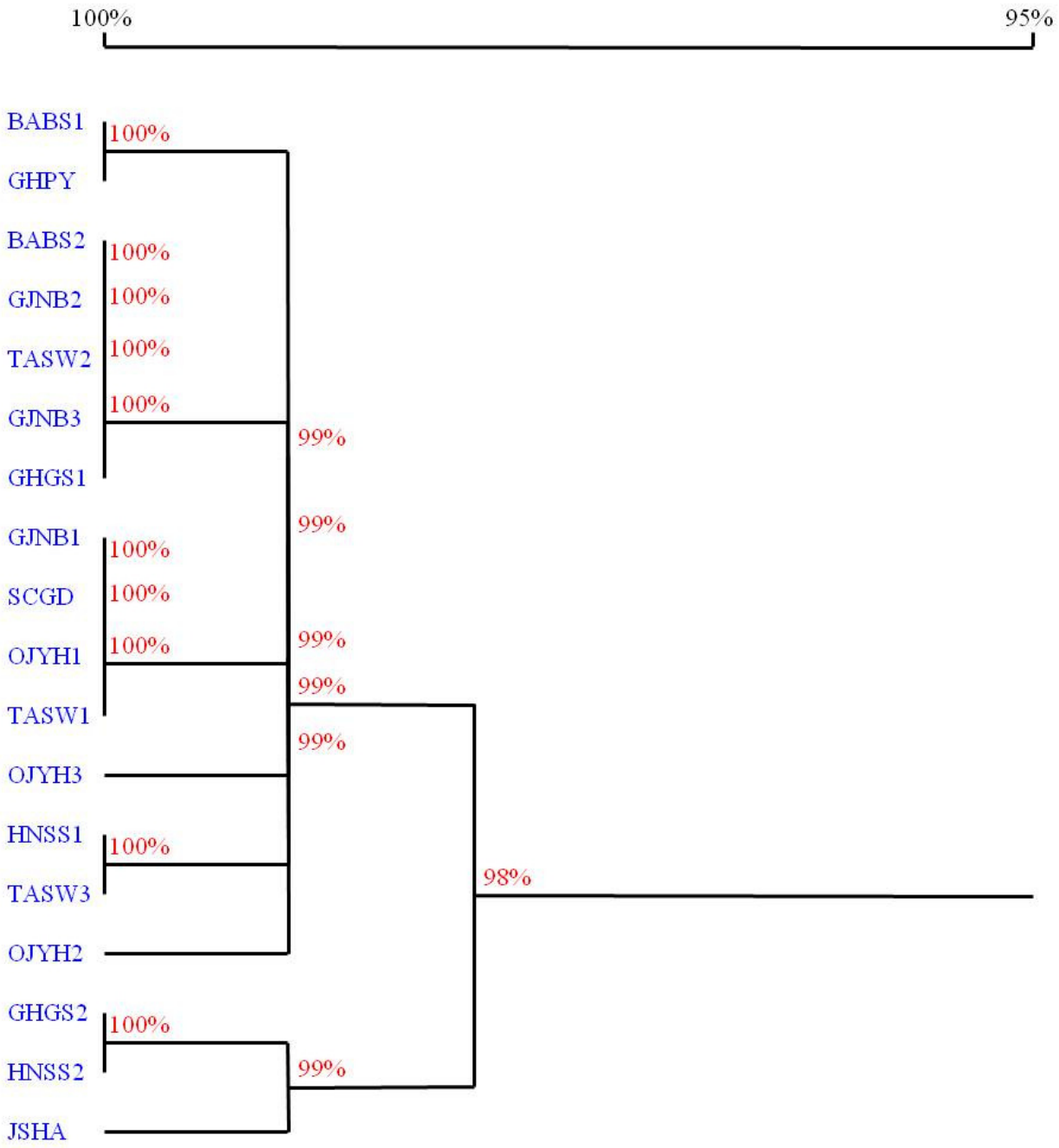

Figure 1. Infraspecies phylogenetic tree of eighteen isolates of *Carpinus turczaninovii* according to Observed Divergency distance method analyzed by DNAMAN 6.0 software.

of *C. turczaninovii* in Korea based on the total ITS sequences. The infraspecies phylogenetic analysis was found to not be correlative with their geographical distribution absolutely, such as GHGS2, HNSS2 and JSHA were collected from different geo-graphical origins, but formed one monophyletic clade. So, this result suggest that *C. turczaninovii* isolates in Korea could be separated into several subgroups, but with relatively high similarity.

Yoo and Wen (2002) performed a morphological analysis based on 23 characters of inflorescence, nutlet, fruit bract, seed, and leaves, and suggested that *Carpinus cordata*, *Carpinus fangiana*, and *C. japonica* composing section *Distegocarpus* formed a paraphyletic group, and section *Carpinus* is monophyletic. Our results support section *Distegocarpus* was paraphyletic, but not the later. *C. fangiana* and *C. japonica* belonging to section *Distegocarpus* formed one group with *C. coreana*

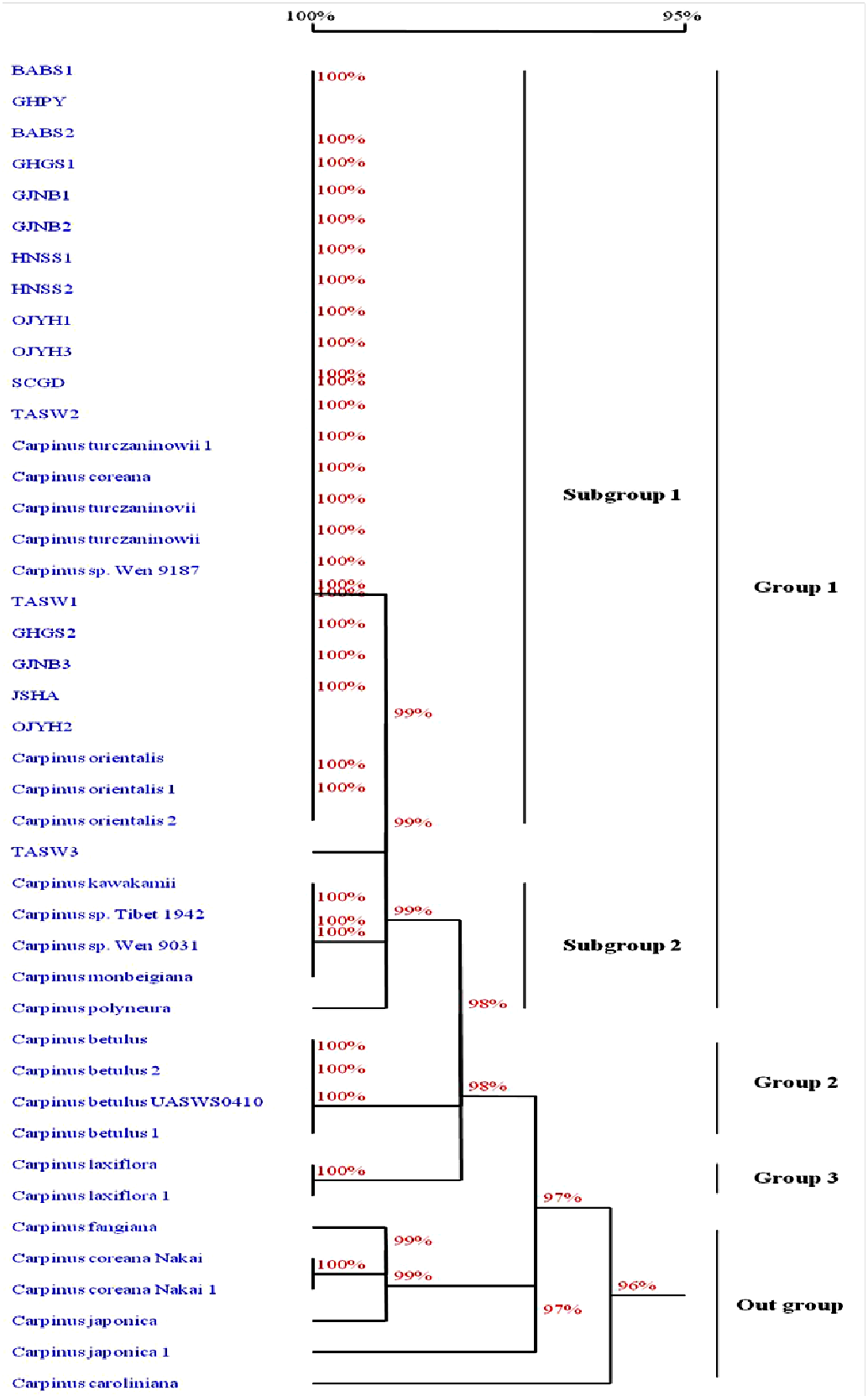

Figure 2. Infrageneric phylogenetic tree of twenty-five known *Carpinus* plant species and eighteen *C. turczaninovii* isolates according to observed divergency distance method analyzed by DNAMAN 6.0 software.

belonging to section *Carpinus*, and combined with *C. caroliniana* also belonging to section *Carpinus* were considered as out group.

C. coreana was firstly reported to be endemic to Korea (Nakai, 1926), and similar to *C. turczaninoii*. However, *C. coreana* and *C. turczaninovii* were very different from each other on morphological characters, including long hairs on the midvein, inflorescences, and leaf margin veins (Nakai, 1926). These morphological differences were also supported by ITS sequence analysis in our study, that *C. coreana* was relatively far from *C. turczaninovii* in the phylogenetic tree.

Our result was congruent with the report by Yoo and Wen (2002) that *C. turczaninowii*, *C. orientalis*, *C. monbeigiana* and *C. polyneura* were monophyletic group based on morphological characters and ITS tree. However, the result that *C. coreana* was also deemed to be in the same monophyletic group with *C. turczaninovii* was not identified with our results.

In addition, *C. caroliniana* and *C. betulus* were thought to be monophyletic compared *C. turczaninowii* in the report by Yoo and Wen (2002) according to the morphological data, while their ITS sequence analysis and our results were congruent that *C. betulus* formed one clade distinct from *C. turczaninovii* group.

Compared to the conventional morphological methodology, molecular classification shares more advantages, example molecular technique is independent of phenotypic and environmental variations, and could be performed using pinpointing plant materials; simple DNA cloning and sequencing might more rapidly provide more phylogenetic information; the information masked by homoplasy of DNA sequences is exacter and more efficient. Thus, this study based on rRNA ITS sequences orientates *C. turczaninovii* in Korea in the phylogenetic tree of the genus *Carpinus* and *C. turczaninovii*, that could help further understanding of clearer classification of *C. turczaninovii* and *Carpinus* species.

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