

Phylogenetic Study of Genus *Sorbus* in Korea by Internal Transcribed Spacer Sequence (ITS)

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Genus *Sorbus* is a long lived woody species that is primarily distributed throughout Asia and Europe. This species is regarded as very important herbal medicines in Korea and China. *Sorbus commixta* is primarily distributed throughout Europe. We evaluated a representative sample of the four taxa with nuclear ribosomal DNA internal transcribed spacer sequences (ITS) to estimate genetic relationships within genus. Aligned nucleotide sequences of the length of ITS1 were nearly constant within genus *Sorbus* varying from 219 in *S. aucuparia* to 218 in the rest species. Especially, the 5.8S subunit of all taxa of *Sorbus* was found to constant of 165 bp nucleotides. However, aligned nucleotide sequences of the length of ITS2 vary from 240 in *S. sambucifolia* var. *pseudogrillo* 245 in *S. aucuparia*. Total alignment length is 629 positions, of which 35 are parsimony-informative, 32 variable but parsimony-uninformative, and 552 constant characters. The base furtherance showed the difference to the by a total taxon: an average A and T are 17.7% and G and C are 30.4%, 34.2%, respectively. All the four taxa beginning with conserved base paired triplets emerging from single strand regions (domain I). Noteworthy, in the RNA secondary structure proposed for the three Korean *Sorbus* taxa RNA transcript ITS2, which shows a remarkably well-conserved folding (domain II). When compared to the European *Sorbus* (*S. aucuparia*) of ITS2. ITS analysis may be useful in germ-plasm classification several taxa of genus *Sorbus*.

Key words : *Sorbus*, ITS, phylogenetic analysis

Introduction

Genus *Sorbus* is known as about 100 species slave-servants range comprehensively over subarctic zone from temperate zones of The Northern Hemisphere. The genus *Sorbus* in Asia is compose of three species and 12 variants [13]. Plants of this genus have been utilized from the example by various usage of coronal shape, industry, edibility, medical use, and so on. Many plants of genus *Sorbus* had been used from the example for good materials for stick and according as new research and practical use way about medical use and so on are risen, as the availability also is increased, the importance and edibility as resources, development value of medical use resources is taken another look at but distribution area ranges natively in high mountain area normalcy department belonging to comparative low temperature land rent and there is special quality that grow and wide research that is research and genetic variation and classification school register organ-

ization process of stock and raw meat environment and physiology, mode of life enemy is much unreciprocated status.

Specially, nutrition effects had been known as water in which medicine has been dissolved that is in discharge of phlegm, bronchial catarrh and several diseases such as body weakness and research about abstraction of material and so on, separation is reported on several kind of flavonoids, carotene and antioxidant [4,12].

Although molecular and biochemical approaches are now increasingly being applied to address the taxonomic and phylogenetic relationships within the animals and plants in Korea, no population genetic studies of genus *Sorbus* have been conducted. In addition, the taxonomy of genus *Sorbus* has processed mainly through morphological characteristics and ecological study [3].

Nuclear ribosomal DNA internal transcribed spacer sequences (ITS) is eukaryotic ribosomal RNA genes (known as ribosomal DNA or rDNA) are found as parts of repeat units that are arranged in tandem arrays, located at the chromosomal sites known as nucleolar organizing regions (NORs) [1]. Each repeat unit consists of a transcribed re-

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gion (having genes for 18S, 5.8S and 26S rRNAs and the external transcribed spacers i.e. ETS1 and ETS2) and a non-transcribed spacer (NTS) region. In the transcribed region, internal transcribed spacers (ITS) are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2.

The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving and therefore may vary. Universal PCR primers designed from highly conserved regions flanking the ITS and its relatively small size (600-700 bp) enable easy amplification of ITS region due to high copy number (up to 30000 per cell) of rDNA repeats [5]. This makes the ITS region an interesting subject for evolutionary and phylogenetic investigations [3,8,9] as well as biogeographic investigations [2,10,15].

The purpose of this research wished to compare with other flora that verify genetic variety of genus *Sorbus* taxa and mass of differentiation whether is possible by ITS in Korea and have genus *Sorbus* paper resemblant life history in the South Korea.

Materials and Methods

Plant materials and DNA extraction

All of the 60 plants were collected from five populations in Korea (Table 1). One young leaf per mature tree (≥ 5 yr) was sampled. Seven or eight plants were randomly collected from each population. In addition, European species, *Sorbus aucuparia*, of the same family Rosaceae was provided for the outgroup and used to compare the phylogenetic relationship.

The genomic DNA of the samples including outgroup was extracted from fresh leaves using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, U.S.A.) according to the manufacturer's protocol.

ITS analysis

Primer sets of about 20 bases in length (ITS1 and ITS2) [17] were used for PCR analysis (Table 2). These primers

Table 2. Synthetic primers used for sequencing the ITS gene in genus *Sorbus* taxa

No.	Primer (5'-3')	Reference
ITS1	TCCGTAGGTGAACCTGCGG	White <i>et. al.</i> (1990)
ITS2	GCTGCGTTCATCGATGC	White <i>et. al.</i> (1990)
ITS3	GCATCGATGAAGAACGCAGC	White <i>et. al.</i> (1990)
ITS4	TCCTCCGCTTATTGATATGC	White <i>et. al.</i> (1990)

were based on well-characteristic DNA Sequences and were designed making use of conserved regions of the 18S and 28S rRNA genes to amplify the noncoding regions between the (ITS1 and ITS2) and 5.8S rRNA gene.

PCR materials (50 ul volume) included 50 ng of genomic DNA, 100 uM of each dNTP, 0.2 uM of each primer, 1 x enzyme buffer, and 2 unit of Taq polymerase. The amplification profile was 28 cycles of 94°C for 30 sec, 42°C for 60 sec, 72°C for 60 sec, preceded by an initial denaturation at 94°C for 90 sec and followed by a final extension at 72°C for 5 min.

PCR products were separated on 1.5% agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN). The amplified fragments were cloned into a bluescript vector and sequenced using ABI Prism 377 Sequencer (Applied Biosystem, USA). At least ten individuals' clones of each taxon were analyzed.

Phylogenetic analysis and RNA Secondary Structure

A pairwise alignment was calculated using the Clustal X program. Phylogenetic relationship were estimated by MEGA4 version 4.0 [16] treating all alignment gaps as missing. A maximum parsimony tree (MP) was inferred using heuristic search, branch-swapping options and tree bisection-reconnection. Confidence values for individual branches were determined by a bootstrap analysis with 100 repeated sampling of the data. In addition, a phylogenetic tree was constructed by the neighborjoining (NJ) method [14] using the NEIGHBOR program in PHYLIP version 3.57 [6]. The ITS structure is use the Vienna RNA Secondary Structure Prediction.

Table 1. Codes and locations of *Sorbus* species used in this study

Taxa	Localities	Code
<i>Sorbus. aucuparia</i>	Osan, Gyeonggi-do	AUC
<i>S. amurensis</i>	Ulleung-do, Gyeongsangbuk-do	AMU
<i>S. sambucifolia</i> var. <i>pseudogracilis</i>	Mt. Gebang, Pyeong chang, Gangwon-do	SAM
<i>S. commixta</i>	Mt. Giri, Sancheong-gun, Gyeongsangnam-do	COM-1
<i>S. commixta</i>	Mt. Gariwang, Jeongseon, Gangwon-do	COM-2

Results

ITS profiles for four *Sorbus* taxa were shown in Figure 1. DNA sequencing of ITS1, 5.8S, and ITS2 were successful in all of the species given in Table 3, but sequencing of 18S failed in two species and was partly successful in another species.

Aligned nucleotide sequences of the length of ITS1 were nearly constant within genus *Sorbus* varying from 219 in *S. aucuparia* to 218 in the rest species. Especially, the 5.8S subunit of all taxa of *Sorbus* was found to constant of 165 bp nucleotides. However, aligned nucleotide sequences of the length of ITS2 vary from 240 in *S. sambucifolia* var. *pseudogracilis* to 245 in *S. aucuparia*. Total alignment length is 629 positions, of which 35 are parsimony-informative, 32 variable but parsimony-uninformative, and 552 constant characters.

The base furtherance showed the difference to the by a total taxon: an average A and T are 17.7% and G and C are 30.4%, 34.2%, respectively. The base furtherance of G+C was showed about 64.6%. The content of C is most high to 36.3% in *S. aucuparia*. The content of G showed about 31.2% averages in Korean spices. However *S. aucuparia* showed about 27.9% (Table 4).

Clustering of *Sorbus* Species, using the NJ algorithm, was performed based on the matrix of calculated distances (Fig. 2). The phylogenic tree showed Korean populations were well separated each other. The tree also shows genetic differentiation among local populations for Korean

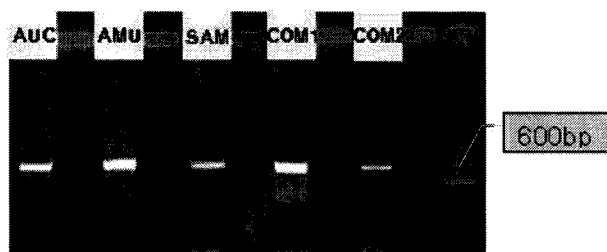


Fig. 1. ITS profiles of genus *Sorbus*.

Table 3. Sizes of ITS1, 5.8S and ITS2 regions of genus *Sorbus*

Taxa	ITS1	5.8S	ITS2	Total
<i>S. aucuparia</i>	219	165	245	629
<i>S. amurensis</i>	218	165	241	624
<i>S. sambucifolia</i> var. <i>pseudogracilis</i>	218	165	240	623
<i>S. commixta</i>	218	165	241	624

species.

All ITS trees generated exhibited partly unsolved topology with low bootstrap support irrespective of the methods (parsimony or N-J) and the setting used. This result confirmed two clades for all species. *S. amurensis* was similar to *S. commixta*, while *S. aucuparia* was more distinct.

S. amurensis, *S. commixta*, and *S. sambucifolia* var. *pseudogracilis* are conform one group and *S. aucuparia* is another. *S. aucuparia* was supported with bootstrap values of 70%.

Interestingly, all the four taxa beginning with conserved base paired triplets emerging from single strand regions (domain I) (Fig. 3). Noteworthy, in the secondary structure proposed for the three Korean *Sorbus* taxa RNA transcript ITS2, which shows a remarkably well-conserved folding (domain II). When compared to the European *Sorbus* (*S. aucuparia*) of ITS2.

We also observed in the distal portion of domain III regions of sequence homology which are reflected in a conserved pattern of folding in *S. sambucifolia* var. *pseudogracilis* *S. amurensis*.

Table 4. Rate of A, C, G, T to four species of genus *Sorbus*

Codes	A	C	G	T
AUC	17.99	36.30	27.86	17.83
AMU	18.24	33.12	30.72	17.92
SAM	17.66	33.23	31.62	17.50
COM	18.29	34.19	31.30	17.50
Mean	17.69	34.21	30.37	17.68

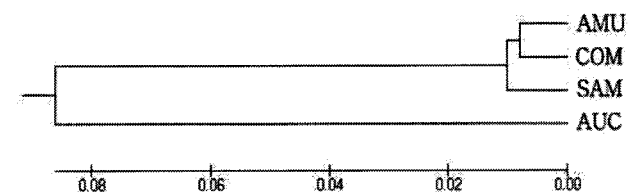


Fig. 2. A phylogenetic tree for genus *Sorbus* based on ITS analysis.

Table 5. Genetic identity (upper diagonal) of four species genus of *Sorbus* taxa based on ITS and genetic distances (low diagonal) at species level

Species	AUC	AMU	SAM	COM
AUC	-	83.1	84.1	83.3
AMU	19.2	-	98.1	98.4
SAM	17.9	1.9	-	98.3
COM	19.0	1.6	1.7	-

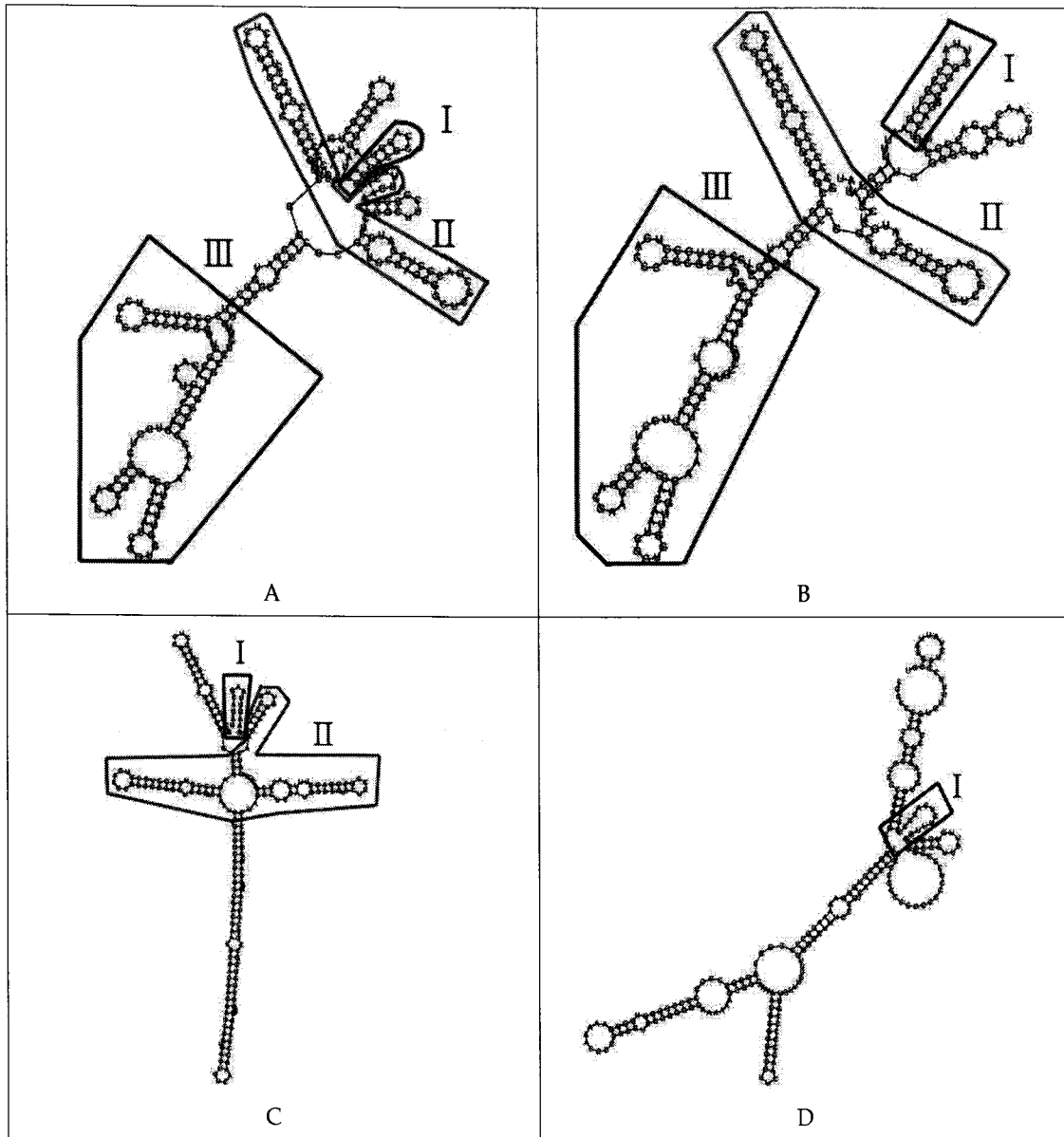


Fig. 3. The results ITS RNA secondary structure prediction of genus *Sorbus* using the Vienna RNA package. A: *S. sambucifolia* var. *pseudogracilis*, B: *S. amurensis*, C: *S. commixta*, D: *S. aucuparia*.

Discussion

Given the proliferation of genetic markers, comparisons between techniques are inevitable. However, there is a need technique is best suited the issues being examined. In previous study, RAPDs were used to determine the genetic relationships among populations and the results compared to pedigree relationships where there were available [13]. In addition, from the dendrogram of the species studied, a very good agreement with the existing classification based on morphology and biochemical studies of the species of

genus *Sorbus* was observed [11]. First, *S. sambucifolia* var. *pseudogracilis* grouped with one of its possible parent species of *S. commixta*. Secondly, *S. aucuparia* which is imported from European rowan is separated from the remaining Korean rowan clades. Finally, although both outgroups of *S. alnifolia* and *S. sorbifolia* are same genus *Sorbus* and grouped together, they did not include true members of rowan.

Common life history traits, such as wind dispersal or animal dispersal of both pollen grains and seeds, high reproductive capability, similar longevity and successional

behavior, could more readily account for most of the homology in the population genetics of these species, and most likely for the low differentiation observed at the intraspecific level. These factors can reduce the effect of geographic isolation for genetic divergence.

Sorbus aucuparia is mostly distributed in Europe and America [4]. This species is introduced in Korea about one hundred years ago which today is widely appreciated ornamental and landscape architecture.

The *S. commixta* and *S. aucuparia* were morphologically distinguished from each other [4]. One of the most striking features between both taxa was length and diameter of winter buds. However, the resolving power of morphological characteristics is restricted, mainly because of the small number of characters available. Efficient methods to clarify the taxonomic status of several species are much needed. The molecular data allowed us to resolve well-supported clades in Korean taxa and European species.

By current categories for threatened taxa in Korea, all species belonging to genus *Sorbus* are not considered threatened. The probability of extinction of any single population is high, since the populations or populations of natural *S. commixta* are so small. Ecological management of these populations will be necessary to preserve species. Many populations of genus *Sorbus* are decline in population size because of the habitat loss by such road-structuring, land development business in a low mountain, and human activities such as over-gathering medicinal plants. Taking the circumstances into this consideration, some populations showed the highest genetic diversity by DNA fingerprinting, thus these populations may be recommend for in-situ conservation.

There is a good agreement between results from ITS sequence analysis and RAPD [13], although the ITS1/5.8S regions do not show enough resolution potential among several species. The ITS technique is more useful taxonomic tool for genus *Sorbus* species and species delineation than the ITS sequences.

References

- Adams, R. P., A. E. Schwarzbach and R. N. Pandey. 2003. The concordance of terpenoid, ISSR, and RAPD markers, and ITS sequences data sets among genotypes: an example from *Juniperus*. *Biochem. System. and Ecol.* **31**, 375-387.
- Baldwin, B. G. 1993. Molecular phylogenetics of *Calcydenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: Chromosomal and morphological evolution reexamined. *Am. J. Bot.* **80**, 222-238.
- Baldwin, B. G., M. J. Sanderson, J. M. Porter, M. F. Wojciechowski, C. S. Campbell and M. J. Donoghue. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Mo. Bot. Gard.* **82**, 247-277.
- Choi, M. S. 2004. Taxonomic and Ecological Study on the Genus *Sorbus* in Korea. pp. 97, Kangwon National Univ., Ph. D. thesis.
- Dubouzet, J. G. and K. Shinoda. 1999. Relationships among old and New world *Alliums* according to ITS DNA sequence analysis. *Theor. Appl. Genet.* **98**, 422-433.
- Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5s, Distributed by the author. Department of Genetics, Univ. Washington, Seattle.
- Hamrick, J. L., M. J. W. Godt and S. L. Sherman-Broyles. 1992. Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6**, 95-124.
- Hershkovitz, M. A. and E. A. Zimmer. 1996. Conservation patterns in angiosperm rDNA ITS2 sequences. *Nucleic Acid Res.* **24**, 2857-2867.
- Hershkovitz, M. A., E. A. Zimmer and W. J. Hahn. 1999. Ribosomal DNA sequences and angiosperm systematics, pp. 268-326, In Hollingsworth, P. M., R. M. Bateman and R. J. Gornall (eds.), *Molecular Systematics and Plant Evolution*, Taylor & Francis, London.
- Hsiao, C., N. J. Chatterton, K. H. Asay and K. B. Jensen. 1994. Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. *Genome* **37**, 112-120.
- Kim, S. H., M. K. Huh and J. H. Lee. 2007. Genetic relationships of among taxa of genus *Sorbus* based on ISSR marker analysis. *KMiskininkyste* **61**, 69-75.
- Na, M. K. 2000. Antioxidant compositions from the bark of *Sorbus commixta* Hedlund. pp. 75, Chungnam National Univ., MS. thesis.
- Park, S. H. S. H. Kim, H. W. Seo and M. K. Huh. 2006. Study of genetic diversity and taxonomy of genus *Sorbus* in Korea using random amplified polymorphic DNA. *Korean J. Life Sci.* **17**, 470-475.
- Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406-425.
- Suh, Y., L. B. Thien, H. E. Reeve and E. A. Zimmer. 1993. Molecular evolution and phylogenetic implications of ribosomal DNA in Winteraceae. *Am. J. Bot.* **80**, 1042-1055.
- Tamura, K., J. Dudley, M. Nei and S. Kumar. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596-1599.
- White, T. J., T. Bruns, S. Lee and J. Taylor. 1999. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics, pp. 315-322, In Innis M. A., D. H. Gelfand, J. J. Sninsky and T. J. White (eds.), *PCR Protocols: A Guide to Methods and Applications*, New York Academic press.

초록 : ITS에 의한 한국내 마가목 속 분류군의 유전적 계통분류학적 연구허만규* · 김세현¹ · 박소혜(동의대학교 분자생물학과, ¹임업연구원 산림유전자원부)

마가목 속(genus *Sorbus*)은 목본류로 아시아와 유럽에 분포되어 있다. 마가목 속 식물은 한국과 중국에서 약용으로 쓰인다. 한국내 마가목 속 식물에 대해 ITS에 의한 계통관계를 조사하였다. 마가목 속 전체 종에서 5.8S exon은 165 핵산서열이었다. ITS1은 유럽마가목(*S. aucuparia*)에서 218 핵산서열인 반면 한국 내 마가목 종은 219 핵산서열이었다. ITS2 핵산서열은 다양하였는데 *S. sambucifolia* var. *pseudogrclisto*에서는 240 핵산서열로 가장 적은 반면 *S. aucuparia*에서는 245 핵산서열이었다. ITS 전체 서열은 625개로 35개는 절약법에 정보적이었다. ITS 서열로 한국내 분류군과 유럽 중간 구분이 잘 되었다. RNA 2차 구조 추정 분석에서 도메인 I과 II는 마가목 속에 잘 보존 되어 있는 반면 도메인 III에서는 많은 차이를 나타내었다. ITS 서열로 종 동정에 이용할 수 있었으며, 종의 보전이나 생식질 보전에 기초로 이용될 수 있을 것으로 사료된다.