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Full Length Research Paper

Genetic evaluation of domestic walnut cultivars trading on Korean tree markets using microsatellite markers

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Walnut (*Juglans regia* L.) is regarded as a healthy food because of its high nutritional composition and various health benefits. Although several walnut cultivars are being actively traded for domestic plantation or ornament in Korea, no particular effort has been made to evaluate genetic quality management of walnut cultivars in domestic tree markets. In this study, as an effort to evaluate the status of walnut seedling trade, we collected walnut seedlings of diverse cultivars from domestic tree markets and several locations in Korea and performed genotype analysis for the collections using microsatellite markers (76 individuals belonging to eight domestic cultivars). We used 12 markers that were previously reported to be informative and polymorphic in walnuts. The number of alleles that was detected from the collections using these markers ranged from 6 to 16, with heterozygosity values ranging from 0.03 to 0.75. Dendrogram revealed that the domestic walnut cultivars trading on tree markets could be genotypically distinguished from various foreign cultivars. However, genotyping data also showed that individual plants belonging to identical cultivars were sporadically distributed on the dendrogram, indicating that walnut cultivars trading on domestic tree markets seem to be poorly managed.

Keywords: Walnut (*Juglans regia* L.), microsatellite markers, tree market, seedling trade, domestic cultivars, foreign cultivars, cultivar management.

INTRODUCTION

Walnut (*Juglans regia* L.), belonging to the family Juglandaceae, is a tall deciduous broadleaf tree. It is widely naturalized in temperate and tropical regions including Asia (western, Caucasus, middle and tropical) and southeastern Europe. It has various common names including Persian walnut and English walnut. It is also prized as a multipurpose species, including ornamental,food (nut and oil) for humans and wildlife, industrial materials (dyestuff and timber) and folklore medicines (United States Department of Agriculture-Agricultural Research Service, Germplasm Resources Information Network; http://www.ars-grin.gov/). Since the first introduction of a walnut cultivar into Korea from China approximately 700 years ago, it has become widely cultivated in the south of Pyeongtaek, Wonju and Gangneung provinces (http://www.nature.go.kr/). In Korea, walnuts are widely used as ingredients in various

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food types including drinks and desserts.

Microsatellites are sequences made up of a single sequence motif usually not more than six bases long and tandemly repeated. Other various names have been used to describe the tandemly repeated sequences, including simple sequence repeats (SSRs) and short tandem repeats (STRs) (Hancock, 1999; Ellegren, 2004). Microsatellites appear to be more or less uniformly distributed throughout eukaryotic genomes, showing high allelic diversity due to variable numbers of the tandem repeats, and are inherited in a codominant fashion (Powell et al., 1996).

Various approaches have been used to explore genetic diversity and relationships among species or cultivars, including isozymes (Ouji et al., 2011), restriction fragment length polymorphism (RFLP) (Fjellstrom et al., 1994), randomly amplified polymorphic DNA (RAPD) (Ku et al., 2011) and inter-simple sequence repeat (ISSR) (Yang et al., 2007) markers. Among them, microsatellite markers are characterized by high polymorphism, reproducibility, and an easy and cost-effective manner (Powell, 1996). Microsatellite markers are now widely used for various genetic analyses including cultivar identification, gene flow and parentage analysis, genome mapping, and genetic characterization of germplasm (Ellegren, 2004).

As walnut is accepted as a health-improving food because of its high nutritional composition and excellent antioxidant effects, the market size of walnut products is rapidly increasing in Korea. Although various walnut cultivars for plantation or ornament are being traded in domestic tree markets, no practical efforts have been made to evaluate genetic quality management of walnut cultivars trading on domestic tree markets. Nuclear microsatellite markers have been developed for the genus *Juglans,* including *J. regia* L. (Woeste et al., 2002; Dangl et al., 2005; Hoban et al., 2008), and used for cultivar identification, paternity analysis, gene flow, genetic variation and population structure analysis (Dangl et al., 2005; Bai et al., 2007; Bai et al., 2010; Gunn et al., 2010). In this study, we performed genotype profiling for domestic walnut cultivars using previously developed microsatellite markers for two purposes: 1) to know whether it is possible to genetically distinguish domestic walnut cultivars from foreign cultivars, and 2) to know whether genetic quality management of walnut seedlings trading on domestic tree markets is under tight control.

MATERIALS AND METHODS

Plant material and extraction of genomic DNA

Samples were collected from 76 individuals of eight domestic cultivars of *J. regia* L. Domestic cultivars and the number of plants (in parentheses) used in this study are as follows: Bong Hwa (10), Bong Hwang (10), Gwang Duck (7), Geum Wang (10), Hwang Ryoung (10), Sang Chon (10), Shin Nong (11) and Yo Ryoung (8). One plant belonging to the Gwang Duck group was collected from a natural monument growing in the temple Gwang Duck. Bong Hwa and Sang Chon cultivars were collected from local farms located in Bong Hwa-gun and Sang Chon-myeon, respectively. Seedlings of all other plants were purchased from local tree markets. Leaf or stem tissues were ground in liquid nitrogen, and then total genomic DNA was isolated using the method of Doyle and Doyle (1987). DNA quality and quantity were determined using a 1% agarose gel (Biobasic, Inc., Canada).

Polymerase chain reaction (PCR) amplification and genotyping

Genomic DNA was diluted to 25 ng/ µl in nuclease-free water (Biomedic Co., Ltd., Korea). PCR was conducted using an ABI 2720 thermal cycler (Applied Biosystems, USA) in a total volume of 50 µl containing 50 ng DNA, 1 x FX Taq buffer (Biomedic Co., Ltd.), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM each primer, 1.25 unit FX Taq polymerase (Biomedic Co., Ltd.). The conditions for PCR amplification were as follows: 3 min for initial denaturation at 95°C, 30 cycles of 30 s at 94°C, 30 s at 56 or 60°C, 1 min at 72°C, concluding with 1 cycle of 5 min at 72°C. PCR products were separated in a 1.5% agarose gel to check PCR amplification. PCR primer sets used in this experiment are listed in Table 1. Forward primers were labeled with a virtual dye 5'-FAM (Applied Biosystems). After PCR amplification, 0.2 µl of PCR products were mixed with 9.8 µl Hi-Di formamide (Applied Biosystems) and 0.2 µl of GeneScan™ 500 LIZ® size standard (Applied Biosystems). The mixture was denatured at 95°C for 5 min and placed on ice. The amplified fragments were separated by capillary electrophoresis on an ABI 3730*xl* DNA analyzer (Applied Biosystems) using a 50-cm capillary with a DS-33 install standard as a matrix. We analyzed size of alleles using GeneMapper software (version 4.0; Applied Biosystems) and calculated allele number and heterozygosity.

Data analysis

We calculated the observed number of alleles, effective number of alleles, observed and expected heterozygosity, and observed genotypes using PopGene (version 1.32; Yeh and Boyle, 1997). We used PowerMarker (version 3.25; Liu and Muse, 2005) to calculate polymorphic information content (PIC) (Bostein et al., 1980). A dendrogram based on the genetic distance values was constructed using unweighted pair group method with arithmetic mean (UPGMA) algorithm (Sneath and Sokal, 1973). Genetic distance was calculated using CS Cord distance (Cavalli-Sforza and Edwards, 1967).

RESULTS AND DISCUSSION

To know whether it is possible to genetically distinguish domestic walnut cultivars from foreign cultivars, we merged and analyzed genotype profiling data for 76 individuals belonging to eight domestic cultivars using eight microsatellite markers with the previous allele sizing data of 12 foreign cultivars in the United States, United Kingdom and France (Dangl et al., 2005). Amplified PCR products ranged in size from 140 to 280 base pairs (bp) (Supplementary Table 1). The number of alleles detected per locus varied from 6 (WGA332) to 16 (WGA376), with an average of 11 (Table 2). The observed hetero-zygosity (H_o) of a given locus ranged from 0.16 (WGA004) to 0.61 (WGA376), with an average over all eight loci of 0.38 (Table 2). As an approach to evaluate whether genetic

Table 1. Twelve microsatellite markers used in this study.

Locus name	Primer sequence (5'→3')[c]	Temperature (°C)	Repeat motif	Expected amplicon size range (bp)[d]
WGA002[a]	FAM-GACGACGAAGGTGTACGGAT GTACGGCTCTCCTTGCAGTC	56	(GA)n	169
WGA004[a]	FAM-TGTTGCATTGACCCACTTGT TAAGCCAACATGGTATGCCA	56	(GT)n(GA)n(GA)n	228-241
WGA007[a]	FAM-CAAACAAAATCCGACCGC AAACCTCGATGAGCGAAGAA	56	(GA)n	222
WGA042[a]	FAM-GTGGGTTCGACCGTGAAC AACTTTGCACCACATCCACA	60	(GA)n	241
WGA045[a]	FAM-TCGTTACCACCAGCACAGAG GACATAGCGAGGGGCTAGG	60	(CT)n	233
WGA069[a]	FAM-TTAGTTAGCAAACCCACCCG AGATGCACAGACCAACCCTC	60	(GA)n	158-182
WGA276[b]	FAM-CTCACTTTCTCGGCTCTTCC GGTCTTATGTGGGCAGTCGT	60	(TC)n	143-192
WGA321[b]	FAM-TCCAATCGAAACTCCAAAGG GTCCAAAGACGATGATGGA	56	(GA)n	222-245
WGA331[b]	FAM-TCCCCCTGAAATCTTCTCCT CGGTGGTGTAAGGCAAATG	60	(GA)n	273-277
WGA332[b]	FAM-ACGTCGTTCTGCACTCCTCT GCCACAGGAACGAGTGCT	56	(CT)n	214-225
WGA349[b]	FAM-GTGGCGAAAGTTTATTTTTTGC ACAAATGCACAGCAGCAAAC	60	(CT)n	258-274
WGA376[b]	FAM-GCCCTCAAAGTGATGAACGT TCATCCATATTTACCCCTTTCG	56	(GA)n	218-254

[a]Primer sequences were obtained from Woeste et al. (2002); [b]Primer sequences were obtained from Dangl et al. (2005); [c]Forward primers were presented first; [d]The expected amplicon size range is based on Woeste et al. (2002) and Dangl et al. (2005).

quality management of walnut seedlings trading on domestic tree markets is under tight control, we performed genotyping analysis using 12 microsatellite loci (Woeste et al., 2002; Dangl et al., 2005) for 76 individuals belonging to eight domestic cultivars, which are largely cultivated at walnut farms in Korea. Table 3 summarizes characteristics of 12 microsatellite markers based on 76 individuals belonging to eight domestic cultivars. Allele sizes varied from 140 to 338 bp (Supplementary Table 2). The number of alleles ranged from 4 (WGA007) to 16 (WGA045 and WGA376) across 12 microsatellite loci, with an average of 9.58. The average H_o value was 0.36, ranging from 0.03 (WGA007) to 0.75 (WGA042) (Table 3). The average H_o values obtained from two analyses (Tables 2 and 3) was much lower than the previous value (0.59) from the analysis using 47 Persian walnut accessions (Dangl et al., 2005). This low heterozygosity at several loci is possibly attributable to careless clonal propagation (for example, grafting to rootstocks) of some domestic cultivars during seedling production. Several markers (WGA002, WGA045, WGA331, WGA376, WGA349 and WGA069) failed to make amplification from several individuals, resulting in availability values lower than one (Tables 2 and 3). Failure of PCR amplification might be attributable to mutations on the conserved sites for PCR primers flanking the tandem repeat region, possibly signifying null alleles (Dakin and Avise, 2004).

Dendrograms are an efficient means of summarizing microsatellite data and can reveal relationships, including individuals with identical genotypes. A UPGMA tree was generated based on the genetic distances between 76 individuals of eight domestic cultivars and 12 foreign cultivars, including one hybrid rootstock Paradox

Table 2. Characteristics of 8 microsatellite markers based on 8 domestic and 12 foreign cultivars.

Locus	Allele frequency	N[a]	No. of *Obs.*	No. of Allele	Availability[b]	H_e[c]	H_o[d]	PIC[e]
WGA004	0.88	88	88	11	1	0.23	0.16	0.23
WGA069	0.75	88	87	13	0.99	0.43	0.24	0.42
WGA276	0.26	88	88	12	1	0.82	0.57	0.79
WGA321	0.77	88	88	9	1	0.4	0.26	0.38
WGA331	0.55	88	83	11	0.94	0.65	0.42	0.61
WGA332	0.75	88	88	6	1	0.41	0.3	0.37
WGA349	0.48	88	86	10	0.98	0.72	0.52	0.69
WGA376	0.2	88	86	16	0.98	0.87	0.61	0.85

[a]N, sum of 76 individuals belonging to 8 domestic cultivars and 12 foreign cultivars analyzed previously (Dangl et al., 2005); [b]Availability is defined as 1-*Obs*/N; *Obs*., number of observations; N, number of individuals sampled; [c]H_e, expected heterozygosity; [d]H_o, observed heterozygosity; [e]PIC, polymorphism information content.

Table 3. Characteristics of 12 microsatellite markers based on 8 domestic cultivars.

Locus	Allele frequency	N[a]	No. of *Obs.*	No. of allele	Availability[b]	H_e[c]	H_o[d]	PIC[e]
WGA002	0.78	76	71	8	0.93	0.38	0.1	0.36
WGA004	0.92	76	76	10	1	0.15	0.13	0.15
WGA007	0.97	76	76	4	1	0.05	0.03	0.05
WGA042	0.57	76	76	9	1	0.64	0.75	0.62
WGA045	0.24	76	74	16	0.97	0.87	0.61	0.85
WGA069	0.81	76	75	12	0.99	0.34	0.19	0.33
WGA276	0.3	76	76	8	1	0.78	0.57	0.74
WGA321	0.88	76	76	6	1	0.23	0.2	0.22
WGA331	0.58	76	72	11	0.95	0.63	0.39	0.6
WGA332	0.82	76	76	6	1	0.31	0.24	0.28
WGA349	0.54	76	74	9	0.97	0.65	0.5	0.62
WGA376	0.23	76	74	16	0.97	0.87	0.6	0.85

[a]N, sum of 76 individuals belonging to 8 domestic cultivars; [b]Availability is defined as 1 - *Obs*/N; *Obs*., number of observations; N, number of individuals sampled; [c]H_e, expected heterozygosity; [d]H_o, observed heterozygosity; [e]PIC, polymorphism information content.

'Burbank' (*Juglans hindsii* × *J. regia*) (Dangl et al., 2005). The tree clearly showed that domestic walnut cultivars are genetically different from the foreign cultivars (Figure 1). Interestingly, Lozeronne, a cultivar originating from France, showed closer genetic relationships with some individuals of domestic cultivars (especially, HR01 and SN03) than the other foreign cultivars. Several individuals belonging to Hwang Ryoung and Shin Nong cultivars possibly share pedigree with the French cultivar, Lozeronne (Figure 1). New markers need to be developed to separate Lozeronne cultivar from HR01 and SN03. Mitochondrial DNA analysis is also needed to clearly reveal the genetic relationships among Lozeronne, HR01 and SN03. Two individuals (YR02 and YR04) of Yo Ryoung cultivar were not genetically separated when we analyzed the collections with eight markers. However, we performed genotype profiling for the collections with 12 markers including WGA002, WGA007, WGA042, and WGA045, the individuals were separated, showing a genetic distance with CS Cord value of 0.053 (Figure 2).

The tree also revealed that genetic distances are widely observed even among individuals from identical domestic cultivars (Figure 1), indicating that quality management of walnut seedling trading in domestic tree markets is not under tight control. Such genetic dispersion among individuals of identical cultivars can be attributable to coarse management (for example, seed-mixing) during seedling production by seed distributors.

Open and cross pollination could be one explanation for the genetic dispersion, although the observed heterozygosity was not high (Tables 2 and 3). The genetic mixing among domestic walnut cultivars not only causes severe problems in the production of nuts with uniform quality, but also hinders development of walnut varieties with new traits through the conventional breeding program. Our data further suggest that microsatellite markers are a useful molecular tool for a practical management of cultivar stocks trading in tree markets.

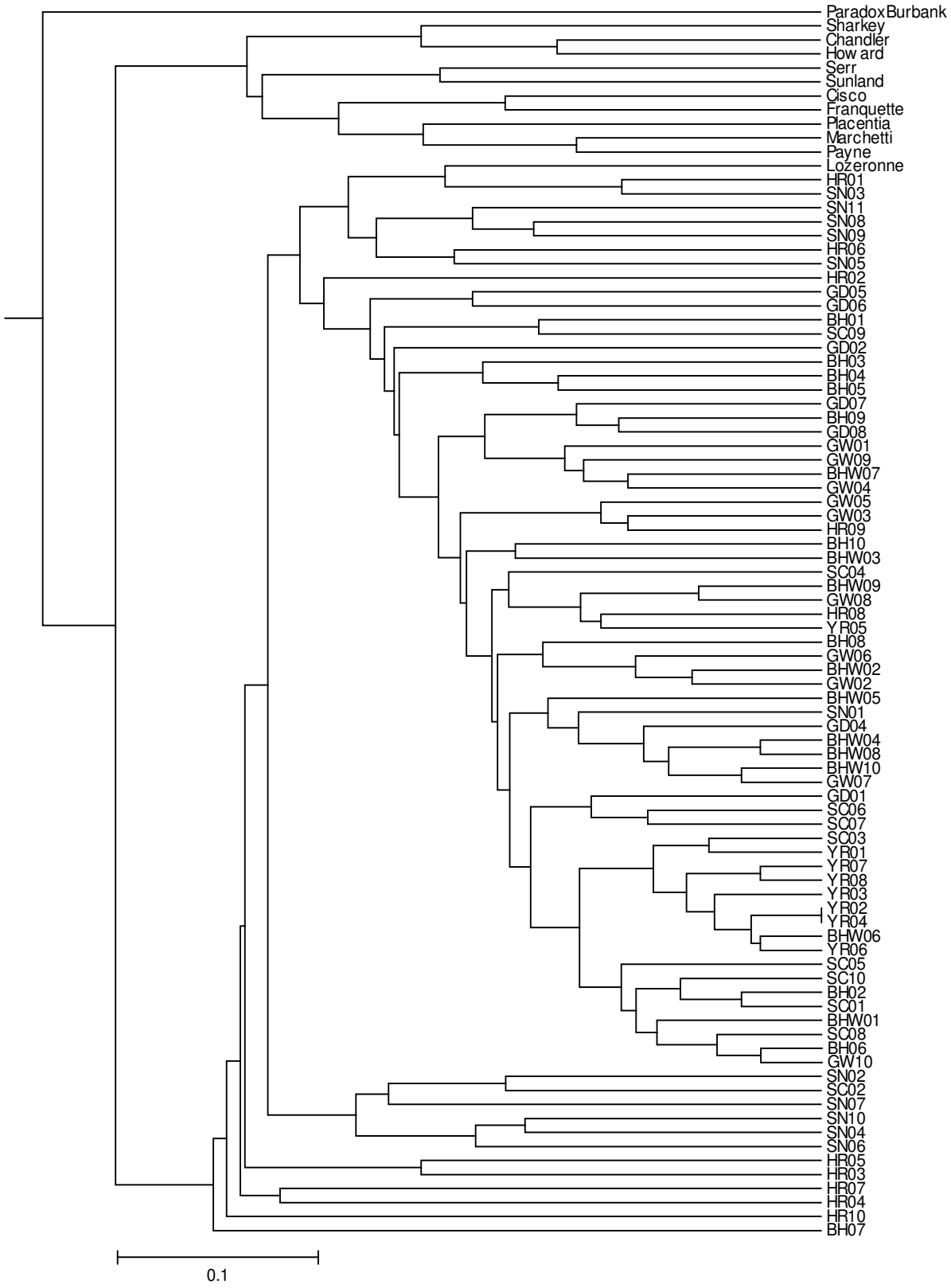

Figure 1. A UPGMA tree based on genetic distances between 76 individuals of 8 domestic cultivars and 12 foreign cultivars including one hybrid (Paradox Burbank), using 8 previously reported polymorphic microsatellite markers (Woeste et al., 2002; Dangl et al., 2005). YR, Yo Ryoung; BHW, Bong Hwang; GW, Geum Wang; GD, Gwang Duck; BH, Bong Hwa; SC, Sang Chon; HR, Hwang Ryoung; SN, Shin Nong.

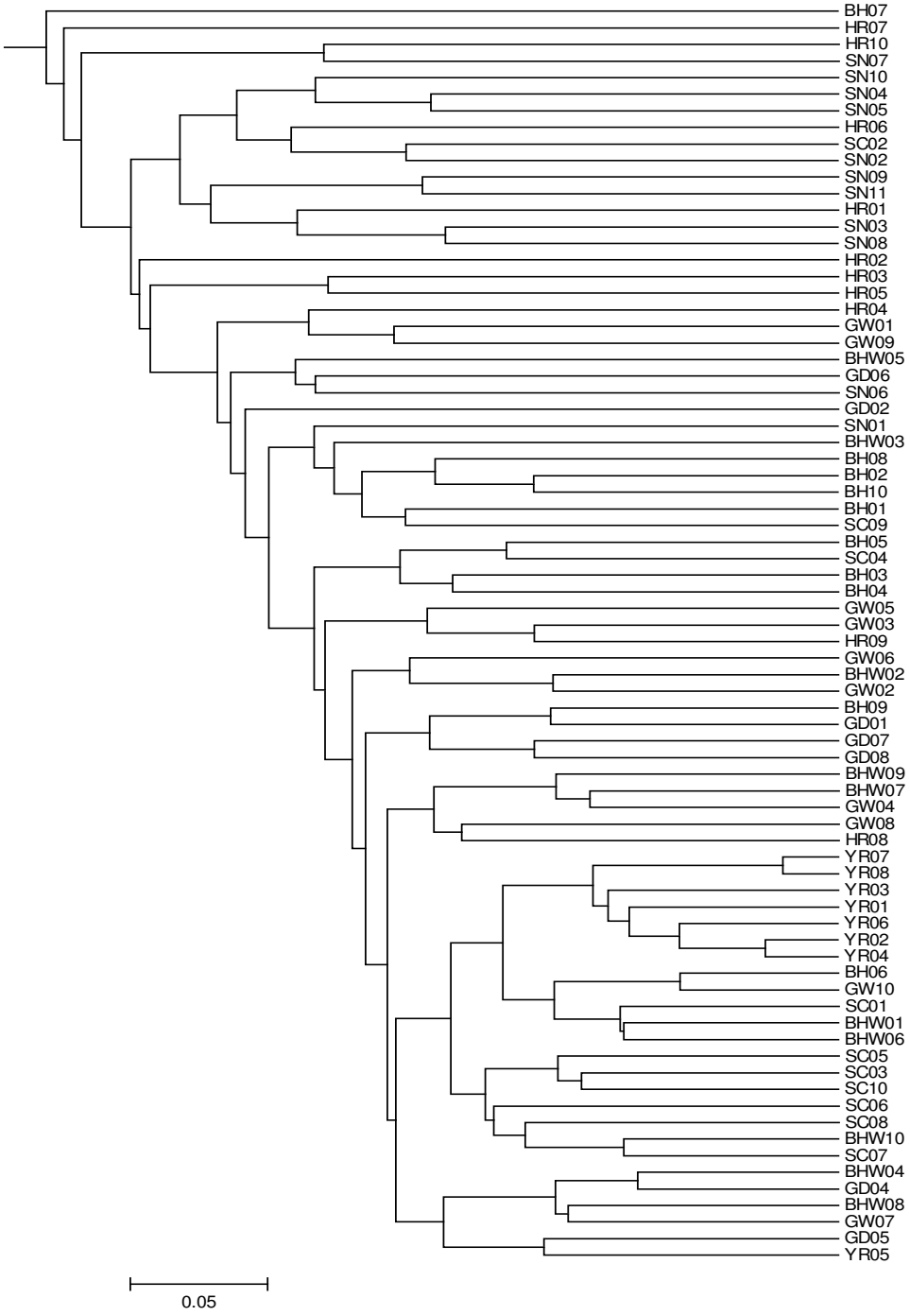

Figure 2. A UPGMA tree based on genetic distances of 76 individuals of 8 domestic cultivars, using previously reported 12 polymorphic microsatellite markers (Woeste et al., 2002; Dangl et al., 2005). YR, Yo Ryoung; BHW, Bong Hwang; GW, Geum Wang; GD, Gwang Duck; BH, Bong Hwa; SC, Sang Chon; HR, Hwang Ryoung; SN, Shin Nong. UPGMA, Unweighted pair group method with arithmetic mean.

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