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ORIGINAL ARTICLE

Phylogeographical Study of *Camellia japonica* Inferred from AFLP and Chloroplast DNA Haplotype Analyses

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Abstract Intraspecific genetic variation provides the information on the distributional pattern of plant species by inducing local adaptation, range shifts, and range reduction. Here, genetic variation of amplified fragment length polymorphism (AFLP) and three chloroplast DNA (cpDNA) regions (*atpI-atpH*, *trnD-psbM*, and *trnT-L*) is investigated in 37 populations of *Camellia japonica* to assess the genetic diversity and population structure. We also infer the phylogeographical history of *C. japonica* distributed in South Korea, Japan (Kyushu and Okinawa), and Taiwan of East Asia. The AFLP results reveal high levels of genetic diversity in *C. japonica* across East Asia. At the regional level, the Kyushu populations display the highest level of genetic variation, whereas the mainland populations of South Korea exhibit the lowest level of variation. Our results show trends of loss of genetic diversity along with latitude. On the basis of 154 polymorphic sites of the combined three cpDNA regions, 11 haplotypes (A-K) were identified across the East Asian *C. japonica* populations. Haplotypes A-C are dominant and widespread in South Korea and Japan, while Haplotypes G, I, and J in Taiwan. In addition, five haplotypes (A, B, D-F) are exclusively occur in South Korea/Japan and five (G-K) are in Taiwan. Our molecular dating analysis estimates the age of initial diversification of *C. japonica* haplotypes in the late Tertiary. The phylogeographic patterns of *C. japonica* coupled with molecular dating suggest vicariance as key mechanism for initial diversification between South Korea/Japan and Taiwan. In contrast, the haplotypes of Japan are shared with those of South Korea indicating that they had insufficient time to form population structures at the regional level.

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Introduction

The distribution range shifts may alter the genetic diversity within species by changing the distribution of genetic variants in the space and time (Milne 2006; Schoettle et al. 2011; Alsos et al. 2012). For example, the level of genetic diversity of North American *Pinus aristata* populations was determined by ecological and geographical parameters such as habitats, latitude, and altitude (Schoettle et al. 2011). The genetic diversity of this species was high in warmer and more humid habitats and its distribution range expanded to the north west in North America. Genetic diversity within a species is crucial for its ability to adapt to environmental and distributional changes (Aitken et al. 2008). Therefore, it is necessary to study genetic diversity and evolutionary history of plant species for understanding the effect of geographic range shifts. However, despite the growing number of studies concentrating on the influences of geographic range change on biodiversity of species and communities (Dieleman et al. 2015; Elmendorf et al. 2015), a few studies focus on patterns of intraspecific genetic variation (Chung and Kang 1994; Schönswetter et al. 2005; Schoettle 2011; Takayama et al. 2013; Han et al. 2015).

The warm temperate climate zones of East China, South Japan, and southern part of South Korea (CJK) in East Asia are characterized by high species richness and endemism (Qian and Ricklefs 2000). Given its remarkable species diversity in such limited area, the CJK region offers an excellent opportunity to study the plant diversification and phylogeographic history. It has been suggested fluctuation in sea level throughout the Quaternary (or even earlier in the late Tertiary) provided

abundant opportunities for population fragmentation and allopatric speciation in the CJK region (Qiu et al. 2011). As for the phylogeographic relationship in the CJK, Xie (1997) suggested that East China (including Taiwan) and South Japan have much closer phylogeographic relationship than South Korea based on the comparison of floristic patterns. Later, molecular phylogenetic studies also support the phylogeographic relationship between East China and Japan (Setoguchi et al. 2006; Li et al. 2008; Qiu et al. 2009a, b). Based on the phylogenetic relationship of two *Kirengeshoma* species (*K. palmata* and *K. koreana*) using cpDNA and inter-simple sequence repeat (ISSR) markers, Qiu et al. (2009b) found a relationship of East China and Japan. They further suggested relatively ancient vicariant divergence of *Kirengeshoma* species in Japan and South Korea at the Plio-Pleistocene boundary [ca. 2.25 million years ago (mya)], whereas segregation of Chinese and Japanese populations occurred during the mid-Pleistocene (ca. 0.45 mya). However, most of these studies have focused on terrestrial herbs in CJK, but few studies have examined the evolution of these disjunct patterns in woody plants.

Camellia japonica L. (Theaceae) is the warm temperate evergreen tree, widely distributed in South Korea, South Japan, and Taiwan (Li 2007) and economically important as ornamentals (Min and Bartholomew 2007). In South Korea, this species grows from northerly Daechungdo (37.8° latitude) to southerly Jejudo (33.2° latitude), but mainly occurs in southern and western coasts of the mainland and islands (Jin 2003). According to previous studies, using allozyme markers, the *C. japonica* populations from South Korea and Japan showed higher level of genetic diversity than other woody plants due to their high life time fecundities, ability to regenerate by stump sprouting, and predominantly sexual outcrossing (Wendel and Parks 1985; Chung and Kang 1996). Later, Ueno et al. (2000) and Lin et al. (2013) also addressed high expected heterozygosity in the Chinese and Japanese populations using microsatellite and ISSR markers. Although earlier studies well documented genetic diversities in the *C. japonica* populations, they evidently could not make inferences on the effect of distribution ranges for genetic variation and phylogeographic history because they focused on the spatial genetic variation in a local population.

The amplified fragment length polymorphism (AFLP) is the one of the most popular techniques to investigate the extent and structure of species genetic variation. Although the AFLP technique has several limitations such as dominance and uncertain locus homology, it has an advantage of surveying the entire genome and providing high level of allelic variability rather than selected fragments. Thus, this has led to their popularity for evolutionary history and conservation applications within a plant species (Karp et al. 1997; Liu and Cordes 2004; Kim et al. 2009). By contrast, chloroplast DNA (cpDNA) is characterized by low mutation rate and maternal inheritance in the angiosperm (Wolfe et al. 1987). Haplotype analyses using cpDNA sequences have been frequently used to investigate the geographic pattern at the intraspecific levels of plants with estimation of divergence time and provide useful insight in the field of phylogeography (Harris and Ingram 1991; Demesure et al. 1996; Ferris et al. 1998; Hewitt 2000; Petit et al. 2002; Lee et al. 2013). Moreover, the genetic relationships of haplotypes could be reconstructed using both conventional dendrograms and nested networks (Lu et al. 2002).

Here, we determine the genetic diversity and structure of *C. japonica* populations in East Asia and ascertain its patterns following geographical information using AFLP markers. We also present a genealogy of cpDNA haplotypes to understand the phylogeographic history of *C. japonica* with inferring the onset of its diversification in East Asia.

Results

Genetic Diversity and Genetic Differentiation

The four primer combinations generated a total of 1105 bands, of which 432 were polymorphic across 537 individuals of 37 *C. japonica* populations (Table 1; Fig. 1). The percentage of polymorphic bands of the four AFLP primer pairs varied from 36.4% (Eco-CATG/MSE-ACGT) to 41.8% (Eco-CAAC/Mse-ACGT), with an average value of 39.0%.

Estimates of genetic diversity at population and species levels are summarized in Table 2. Highly significant correlations

Table 1. Polymorphic bands generated by amplified fragment length polymorphism (AFLP) primer combinations in 37 populations of *C. japonica*

No.	Primer	Total bands	Polymorphic bands	Polymorphism (%)
1	HEX-*Eco*-CAAC / *Mse*-ACGT	277	113	40.8
2	HEX-*Eco*-CACT / *Mse*-ACGT	251	93	37.1
3	6-FAM-*Eco*-CATG / *Mse*-ACGT	283	103	36.4
4	6-FAM-*Eco*-CACC / *Mse*-ACGT	294	123	41.8
	Total	1105	432	-
	Mean	276.3	108.0	39.0

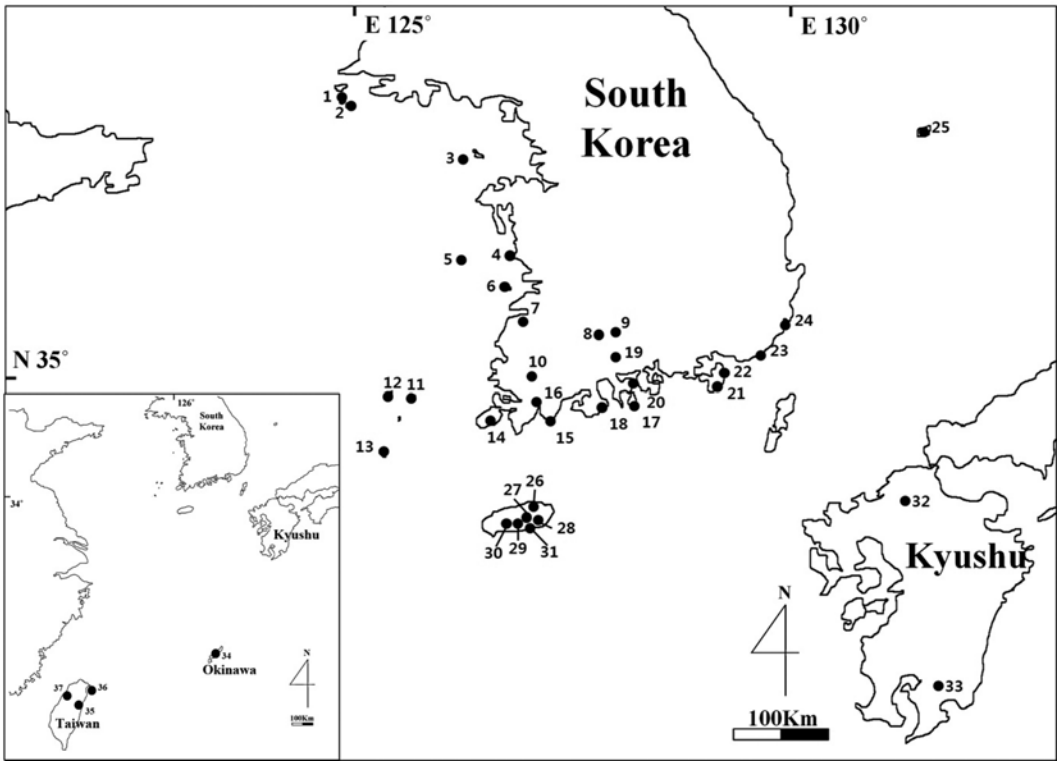

Fig. 1. Collection sites of 37 populations of *Camellia japonica* in South Korea, Japan (Kyushu and Okinawa), and Taiwan (see Table 2 for population codes).

Table 2. Collection details, genetic diversity (H_E), chloroplast DNA haplotypes, and other parameters for 37 populations of *C. japonica*

No.	Population code	No. for AFLP	No. for Haplotype	*PPL* (%)	H_E	Haplotype (number of individuals)	Latitude	Longitude	West coast*
Mainland of South Korea									
1	DC	7	5	51.6	0.187	Type C (5)	37.82	124.69	O
2	SSC	27	5	76.9	0.207	Type C (5)	37.77	124.73	O
3	GU	4	5	41.2	0.171	Type C (5)	37.18	125.95	O
4	MR	10	5	67.6	0.230	Type A (2), Type C (3)	36.13	126.48	O
5	UC	7	5	55.8	0.205	Type C (5)	36.12	125.97	O
6	SS	3	3	36.6	0.163	Type B (3)	35.82	126.45	O
7	SU	7	5	55.1	0.202	Type A (3), Type C (2)	35.48	126.57	O
8	HU	15	6	78.9	0.259	Type A (5), Type B (1)	35.25	127.48	
9	YG	5	5	44.2	0.170	Type A (3), Type B (2)	35.25	127.58	
10	BH	11	5	63.4	0.207	Type A (4), Type B (1)	34.90	126.82	O
11	HK	40	5	83.6	0.235	Type A (4), Type C (1)	34.67	125.40	O
12	HD	36	5	84.0	0.233	Type A (2), Type C (3)	34.68	125.18	O
13	GG	30	5	85.7	0.245	Type A (2), Type B (3)	34.07	125.10	O
14	JD	15	5	75.7	0.242	Type C (5)	34.47	126.30	O
15	JY	5	5	44.9	0.173	Type C (5)	34.37	126.92	
16	BR	16	5	69.4	0.221	Type B (5)	34.58	126.73	
17	HI	7	5	49.3	0.180	Type A (5)	34.58	127.80	
18	NR	7	5	60.4	0.214	Type A (1), Type B (3), Type C (1)	34.47	127.47	
19	OR	16	5	65.5	0.209	Type C (5)	35.03	127.60	
20	OD	9	5	66.0	0.219	Type B (5)	34.73	127.77	
21	HKD	7	5	68.1	0.251	Type A (3), Type C (2)	34.76	128.64	
22	JS	22	5	81.3	0.256	Type B (4), Type C (1)	34.82	128.72	

Table 2. Continued

No.	Population code	No. for AFLP	No. for Haplotype	*PPL* (%)	H_E	Haplotype (number of individuals)	Latitude	Longitude	West coast*
23	PS	13	5	75.2	0.242	Type A (1), Type B (2), Type D (1), Type F (1)	35.08	129.05	
24	MD	48	5	83.1	0.249	Type E (5)	35.43	129.35	
25	UL	28	5	83.6	0.274	Type A (1), Type B (2), Type C (2)	37.52	130.85	
	Mean	15.8	5.0	65.9	0.218				
Jejudo (South Korea)									
26	JJ	8	5	70.8	0.252	Type A (5)	33.48	126.70	
27	JH	7	6	64.6	0.234	Type A (2), Type B (1), Type C (3)	33.30	126.47	
28	JSH	8	5	63.7	0.234	Type A (3), Type C (2)	33.32	126.73	
29	JT1	12	5	69.2	0.228	Type A (5)	33.33	126.65	
30	JT2	13	5	69.0	0.236	Type A (3), Type C (2)	33.30	126.53	
31	JW	11	5	70.4	0.251	Type A (1), Type B (3), Type C (1)	33.27	126.67	
	Mean	9.8	5.2	68.0	0.239				
Kyushu (Japan)									
32	HM	15	5	78.7	0.262	Type A (1), Type B (4)	33.53	130.56	
33	J2	10	6	62.3	0.222	Type B (5), Type D (1)	31.71	131.05	
	Mean	12.5	5.5	70.5	0.242				
Okinawa (Japan)									
34	J1	15	5	69.9	0.227	Type A (5)	26.65	128.14	
Taiwan									
35	T1	12	5	67.4	0.214	Type G (4), Type H (1)	23.94	121.41	
36	T2	18	5	66.0	0.204	Type C (1), Type I (4)	24.84	121.95	
37	T3	13	5	56.3	0.184	Type J (4), Type K (1)	24.35	120.99	
	Mean	14.3	5	63.2	0.201				
Total	Mean	14.5	5	66.4	0.221				
	Species level	537	186	100.0	0.299				

PPL, percentage of polymorphic loci; H_E, Nei's genetic diversity (Nei 1973).
*populations used in correlation analyses between genetic diversity and geographic parameter (latitude).

Table 3. Summary of Analysis of Molecular Variance (AMOVA) for 37 *C. japonica* populations using amplified fragment length polymorphism (AFLP) markers

Source of variation		d.f.	Sum of squares	Variance component	Percentage of variation	p^a
Two hierarchical level	Among populations	36	7882.20	11.56	17.8	<0.001
	Within populations	500	26758.03	53.52	82.2	<0.001
Three hierarchical level	Among regions[b]	4	1558.78	3.31	5.0	<0.001
	Among populations within regions	32	6323.42	10.05	15.0	<0.001
	Within populations	500	26758.03	53.52	80.0	<0.001
	Total	536	34640.22	66.88		

d.f., degrees of freedom
[a]Levels of significance are based on 1000 iteration steps.
[b]Five regions: (1) mainland of South Korea, (2) Jejudo, (3) Kyushu, (4) Okinawa, and (5) Taiwan

were found between amounts of genetic variation (*PPL* and H_E; data not shown). The value of Nei's genetic diversity (H_E) within each population ranged from 0.163 (SS) to 0.274 (UL), with an average of 0.221. At the regional level, the genetic diversity of Kyushu populations (H_E = 0.242) was higher than those of mainland of South Korea (H_E = 0.218), Jejudo (H_E = 0.239), and Taiwan (H_E = 0.201; Table 2).

On the basis of the smallest DIC value, the θ^B = 0.173 was determined to be unbiased estimate of population genetic differentiation under the full model. The AMOVA revealed highly significant ($p<0.001$) genetic differentiation both among and within populations (Table 3). At two hierarchical

levels, 82.2% of total genetic variation was attributed to differentiation within the populations and the remainder (17.8%) to those of among populations. When total variances were partitioned into three hierarchical levels, the largest variance was also observed within populations (80.0%), with 15.0% and 5.0% being found among populations within regions and among regions, respectively (Table 3).

Population Relationships

The NJ dendrogram of 37 populations of *C. japonica* based on Nei's genetic distance revealed two defined groups, representing: (A) the Taiwan (T1, T2, and T3), Okinawa (J1), and South Kyushu (J2) populations; and (B) the remaining populations included the mainland of South Korea, Jejudo, and North Kyushu (Fig. S1). In the admixture analysis using STRUCTURE, the highest likelihood of the data was obtained when samples were clustered into three groups (K =3; Table S1, Fig. 2); (I) T1, T2, T3 (Taiwan), J1 (Okinawa), and J2 (South Kyushu); (II) GU, SS, SU, YG, BH, HK, HD, JD, JY, BR, NR, and OR (mainland of South Korea); (III) the remaining populations of South Korea and HM (Kyushu). The South Korean populations, HU, JH, JW and HKD, JS, PS, JT1 showed evidence of possible introgression between groups I and III and between groups II and III, respectively. Results from the STRUCTURE were largely congruent with those obtained in the NJ analysis except that the JH, JW, and HU populations of mainland of South Korea and Jejudo were grouped with the populations of Taiwan, South Kyushu, and Okinawa. In DAPC analysis, three distinct groups (I-III) with seven clusters were resolved. The groupings of DAPC analysis were entirely consistent with those of the STRUCTURE (Fig. S2).

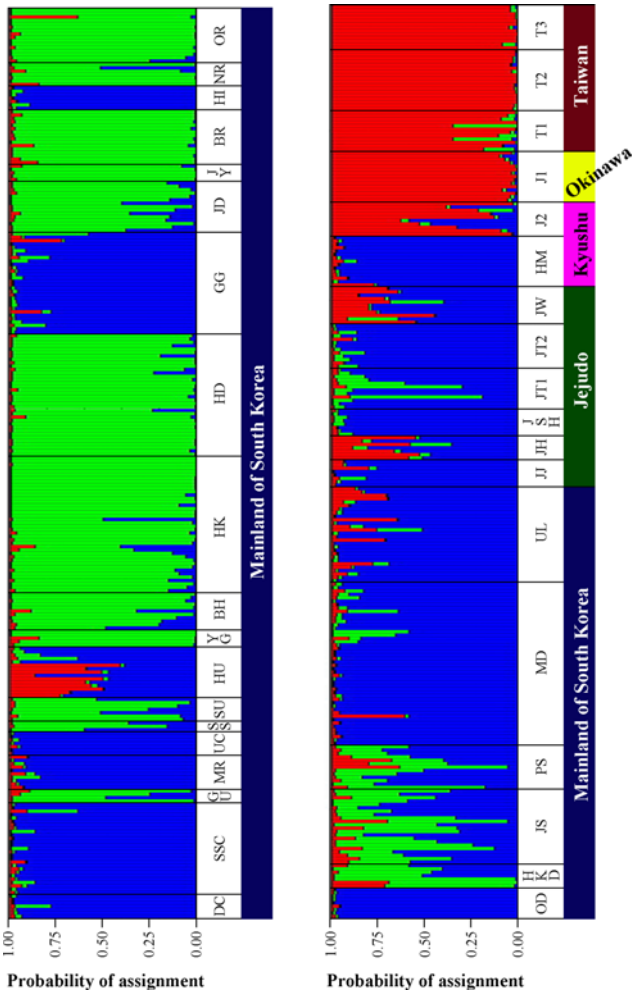

Fig. 2. Bar plot of Ancestry estimates for K = 3, illustrating the genetic composition of the 37 populations of *Camellia japonica* in East Asia using AFLP markers. Blocks indicate 37 populations and each bar represents a single individual (see Table 2 for population codes).

Correlation between Genetic Diversity and Latitude

Highly significant correlations were detected between genetic diversity and latitude in *C. japonica* populations (Fig. 3). The levels of genetic diversity were negatively correlated with the

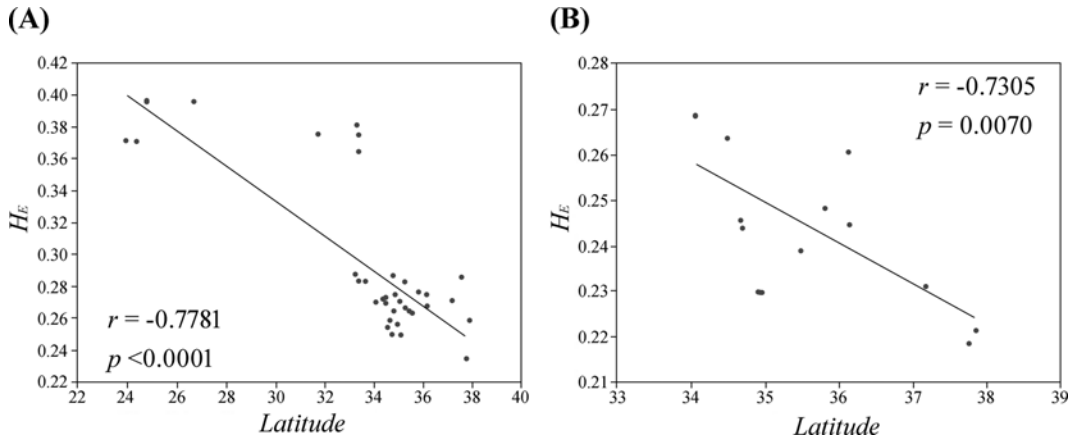

Fig. 3. Scatterplots of genetic diversity (H_E) and geographical parameter of populations of *Camellia japonica.* (A) H_E vs. latitude for all populations. (B) H_E vs. latitude in west coast populations (see Table 2 for west populations information examined).

Table 4. Polymorphic sites and haplotypes based on combined three cpDNA regions (*psbM-trnD, atpI-atpH,* and *trnT-trnL*)

Haplotype	Site															No. of individuals
	psbM-trnD			*atpI-atpH*									*trnT-L*			
	53	397	568	787	812	870	1329	1380	1382	1383	1390 – 1789	1848 – 1864	2226	2430	2587–2596	
Type A	G	G	G	A	T	C	A	G	T	A	*	–	C	G	*	62
Type B	.	A	.	.	.	.	G	.	.	.	*	–	.	T	*	44
Type C	.	.	.	.	.	.	G	.	.	.	*	–	.	.	*	59
Type D	.	A	.	.	.	.	G	.	.	.	*	–	.	.	*	2
Type E	.	.	A	.	.	.	G	.	.	.	*	–	A	.	*	5
Type F	C	.	.	.	.	.	G	.	.	.	*	–	.	.	*	1
Type G	.	.	A	.	A	.	G	.	.	.	*	–	.	.	*	4
Type H	.	.	A	C	A	.	G	.	.	.	*	–	.	.	*	1
Type I	.	.	.	.	.	.	G	.	.	.	*	–	.	.	–	4
Type J	.	.	.	.	.	T	G	T	A	C	–	*	.	.	*	4
Type K	.	.	.	.	.	.	G	T	.	.	–	*	.	.	*	1

All sequences are compared to the reference Type A.
The dot symbol (.) represents a site with the same nucleotide variant as the Type A; "– ", gap; *, multi-nucleotides.

latitude in all populations examined (r = -0.7781, p<0.0001; Fig. 3A) and in the western coast populations of the mainland of South Korea (r = -0.7305, p = 0.0070; Fig. 3B).

Chloroplast DNA Haplotype Analyses

The aligned cpDNA data matrix comprised 2596 characters (*psbM-trnD*, 780; *atpI-atpH*, 1067; and *trnT-L*, 749) and included 9 nucleotide substitutions and 145 indels. On the basis of these polymorphic characteristics, 11 haplotypes were determined (Table 4). Differences between the haplotype C and the haplotypes I and J were caused by indels and the others were led to base substitutions.

The geographic distribution of the 11 haplotypes (A-K) is shown in Fig. 4. Haplotype A (32.8%) and C (31.7%) were dominant in the genetic composition of *C. japonica*, which were shared by 21 and 20 of the 37 populations, respectively. Several populations shared 4 haplotypes (A-D), and other haplotypes (E-K) were unique to a particular population (private haplotype). The distribution of haplotypes was clearly structured between Taiwan and the remaining regions across the geographic range of species. Haplotypes A, B, and C was dominant and widely distributed in the populations of South Korea, Kyushu, and Okinawa, whereas haplotypes G, I, and J was in Taiwan populations. Two haplotypes E and F were only found in South Korean populations, while five haplotypes G-K in Taiwan populations.

Parameters of genetic differentiation were estimated using the PERMUT program. The *Nst* value was significantly higher than the *Gst* value in all populations (*Nst* = 0.972 > *Gst* = 0.562; p < 0.01), suggesting that pairs of different cpDNA haplotypes from same population have more similar sequence.

To clarify the relationships of *C. japonica* populations, a haplotype network was constructed using the TCS program. The 11 haplotypes of *C. japonica* were spilt into two lineages: (1) haplotypes J and K which were only found in Taiwan populations and (2) the remaining haplotypes (A-I). In TCS analysis, haplotype C, which found in South Korea and Taiwan, was suggested as the most probable ancestral type (p = 0.424) for *C. japonica* (Table S2). The Network analysis also indicated haplotype C was positioned in the center that gave rise to other haplotypes (Fig. 5).

The chronogram and results of divergence time estimation of the *C. japonica* haplotypes based on a Bayesian approach applied to the combined cpDNA dataset are shown in Fig. 6. Molecular dating analyses suggested that *C. japonica* diverged from its sister group, *C. oleifera* and *C. crapnelliana*, at 6.3 mya (95% HPD: 2.2–11.3 mya) at the late Miocene/Pliocene interface. The diversification among *C. japonica* haplotypes was estimated to have occurred at 4.9 mya (95% HPD: 2.2–8.6 mya) in the Pliocene.

Discussion

Genetic Diversity and Genetic Differentiation

Using AFLP markers, we determined that *C. japonica* populations had similar or slightly lower level of genetic diversity (mean *PPL* = 66.5% and mean H_E = 0.222) compared with previous studies of *C. japonica* (Wendel and Parks 1985; Chung and Kang 1996; Lin et al. 2013) but, higher than its congeners (Cao et al. 2003; Tang et al. 2006) distributed in East Asia. For examples, Wendel and Parks (1985) determined the genetic diversity in Japanese populations of *C. japonica* to be *PPL* = 66.2%. Tang et al. (2006) have

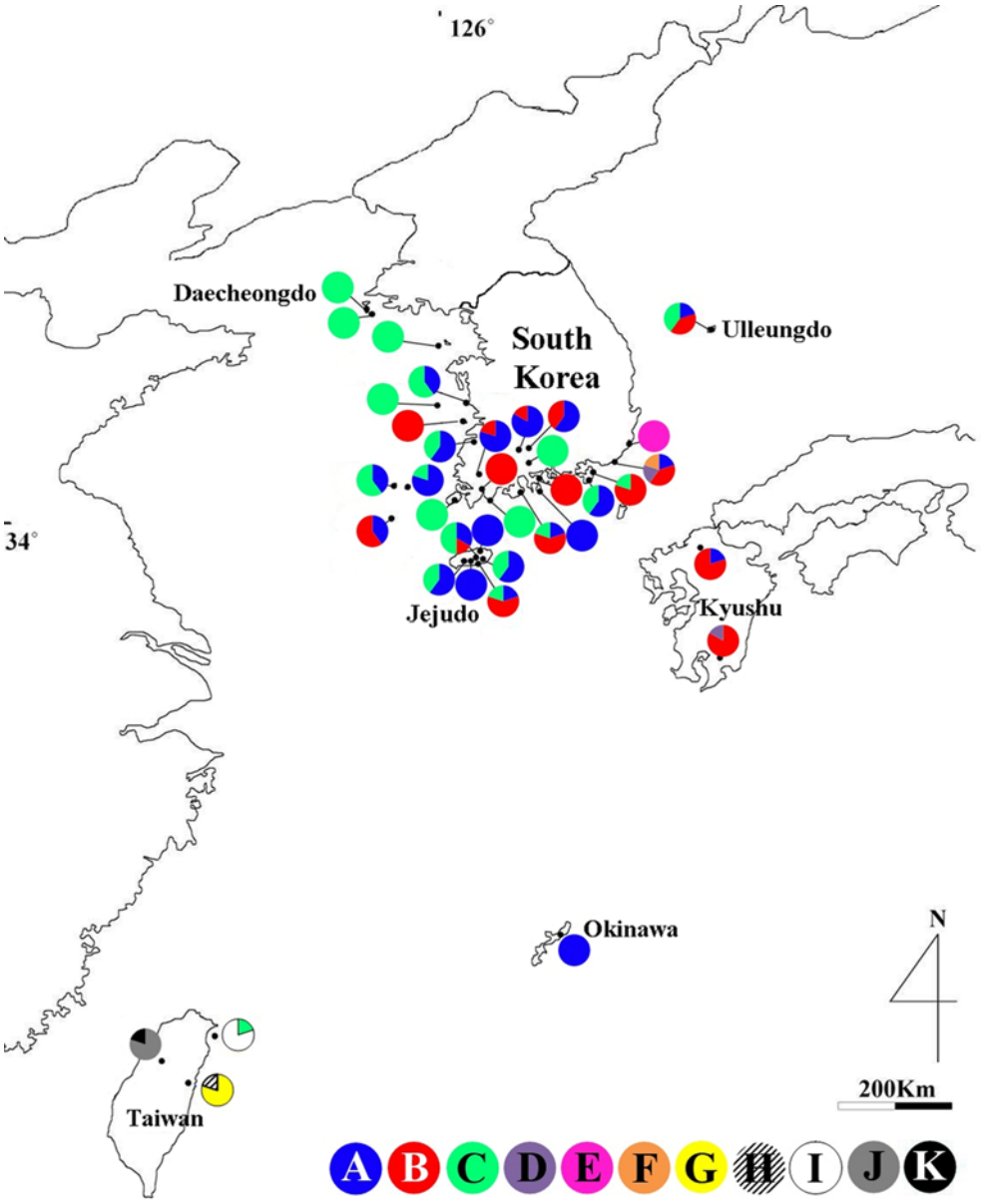

Fig. 4. Geographic distribution of 11 chloroplast DNA haplotypes identified in 37 populations of *Camellia japonica.* Pie charts display haplotype frequencies in each locality.

reported the population diversity for six wild populations of *C. nitidissima*, distributed in the mainland of China, with a mean of H_E values of 0.129. Aside from broad distribution, long generation times, ability to regenerate by stump sprouting, and predominantly outcrossing by animal vectors of *C. japonica* in East Asia (Wendel and Parks 1985; Chung and Kang 1996; Lin et al. 2013), such high genetic diversity is thought to reflect limited inter-population gene flow, hence allowing higher levels of total genetic diversity to be maintained than in case of high gene flow (Vario et al. 1986; Qiu et al. 2009b). The pollinator of *C. japonica* is the resident bird (*Zosterops japonica*; Brazil 2009) and its seeds are dispersed by gravity and rodents that are only capable of limited foraging distance. This pattern suggests limited inter-population gene flow by pollens and seeds and might significantly reduce the potential for long-distance dispersal at a regional scale. Moreover, STRUCTURE and DAPC analyses show subdivision between the populations of South Korea, Japan, and Taiwan with some overlapping (Fig. 2; Fig. S2), suggesting that geographical distribution play an important role to create genetic structure in *C. japonica*.

Comparison between geographical distribution pattern and accumulated genetic variation during population growth help to explain change of populations by climate change (Setoguchi et al. 2006; Qiu et al. 2009b). Our results indicate trends of decreasing genetic diversity with increasing latitude

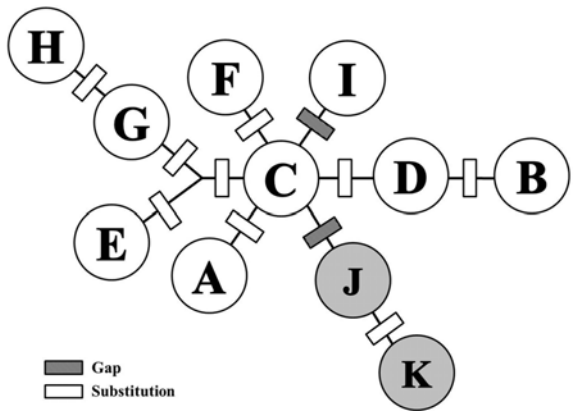

Fig. 5. Relationships among the chloroplast (cp) DNA haplotypes detected in *Camellia japonica* assessed with Network 4.6. One phylogeographic lineage is shown in white, the second in grey.

(i.e. south to north; Fig. 3) in *C. japonica* populations. This finding is consistent with previous studies on Japanese camellia populations (Wendel and Parks 1985) and *Sasa borealis* populations in South Korea (Kim et al. 2015). In general, there is strong correlation between genetic diversity and geographic variables (Mosca et al. 2012). Although latitude itself is not an important factor directly affecting genetic diversity, numerous ecological factors (e.g., temperature and precipitation) that vary with latitude may be responsible for creating latitudinal diversity patterns. The results of previous studies showed the diversity of woody plants increases with decreasing latitude in similar habitats and elevation, with marked declines around the Tropics of Cancer and Capricorn (Schnitzer 2005; Weiser et al. 2007). Although we could not identify the most important factors to

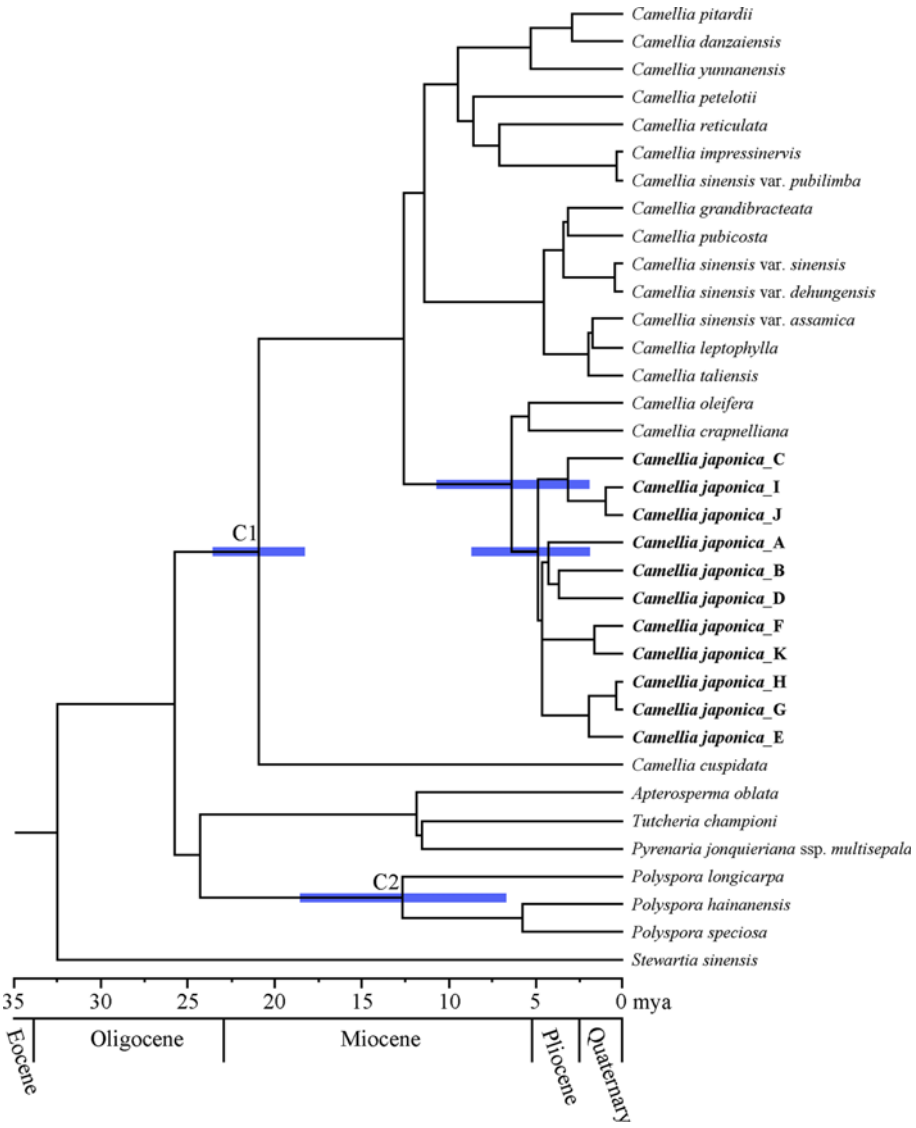

Fig. 6. Chronogram showing the divergence times estimated in BEAST based on the combined three chloroplast DNA regions (*atpI-atpH*, *trnD-psbM*, and *trnT-L*). Blue bars at the nodes of interest represent 95% highest posterior density for the estimated mean dates. Nodes labeled C1 and C2 are the calibration points used in the analysis (for more details, see Materials and Methods).

have an effect on species genetic diversity, the level of genetic diversities of the current *C. japonica* populations might be at least influenced by geographic latitude. Apparently, isolated and small population size play a predominant role in low genetic diversity of northern populations of South Korea. The aspect in relation to conservation and management of *C. japonica* populations in South Korea, the northern populations, mainly grow on island, should be received more attention.

Phylogeographic History

Phylogeographic structure of cpDNA haplotypes in the *C. japonica* populations reflects that populations from South Korea/Japan and Taiwan possesses unique sets of haplotypes with single widespread haplotype (Fig. 4; Fig. 5). The initial diversification of *C. japonica* is estimated to have occurred in the late Tertiary (Fig. 6). Together the phylogeographical pattern and molecular dating analyses imply geographic separation of a formerly widespread ancestral stock into two isolated population groups (Taiwan and the remaining regions). Our results are consistent with the hypotheses that most modern plant species of East Asia originated in the late Tertiary and that an extensive Miocene/Pliocene land bridge was existed between continental Asia and Japan (Ota 1998). Several previous phylogeographical studies in East Asia also indicated that their studied species originated in the late Tertiary (Su et al. 2011; Liao et al. 2016). Aside from the possibility of ancient long distance dispersal in *C. japonica*, we consider a vicariance scenario most likely when considering the ecology of this evergreen tree, which is a constituent of mostly warm-temperate forests in coastal regions and islands. Schoettle et al. (2011), who examined the genetic variation of *Pinus aristata* populations by their geographical distribution, denoted that higher genetic diversities were found in the core populations than in the peripheral ones. The genetic diversities of mainland of South Korea and Jejudo are lower than those detected in Kyushu populations (Japan) (Table 2). Thus, distribution ranges of *C. japonica* populations in East Asia were expanded to the north (South Korea) from low-latitude region (Kyushu, Japan) for suitable growth according to the environment in which the warming progresses.

It is well known that the climate and geological changes during the Quaternary profoundly shaped the current distributions and genetic structures of many plant species in the warm temperate zones of East Asia. Unlikely most plant species in East Asia that appeared to have been diverged in the Quaternary (Li et al. 2008; Qiu et al. 2009a, 2011; Chung et al. 2014), the initial divergence of *C. japonica* haplotypes is estimated to have occurred in the late Tertiary (Fig. 6). Therefore, the geographical events and changing climate conditions before the Quaternary may also be the major factors that resulted in the haplotype divergence within species. In contrast, we did not resolve the South Korean and Japanese populations. That is, all haplotypes occurred in Japanese populations are also detected in South Korean ones (Fig. 4). The nested cladogram of cpDNA haplotypes of *C. japonica* showed star-like clusters that were the results of haplotypes being linked to a central haplotype (Fig. 5). This relatively simple pattern could be explained by populations that had experienced expansion after glaciations but had insufficient time to form a more complicated structure (Dynesius and Jansson 2000; Liao et al. 2016).

Materials and Methods

Sampling and DNA Extraction

For AFLP analysis, we collected a total of 537 individuals of *C. japonica* from 37 wild populations in South Korea (31 populations), Japan (3), and Taiwan (3) from 2013 to 2015 (Table 2; Fig. 1). Sampling locations in South Korea were included through Daechung-do (the northernmost habitat), Gageo-do (the southwesternmost habitat), Ulleung-do (the northernmost habitat in the East Sea), and Jejudo. Depending on the population size, plant leaf materials were collected between 3 and 48 individuals from each population (Table 2). For cpDNA haplotype analysis, 186 individuals representing 37 populations were examined to determine their phylogeographic history. All of leaf tissue was dried in silica gel and stored in -70°C condition before DNA extraction. Genomic DNA was extracted from dried tissue by using a modified CTAB method (Chen and Ronald 1999). The DNA concentration of each sample was determined with a spectrophotometer (Geneflow, Lichfield, UK).

AFLP Analysis

AFLP analysis was performed as described by Vos et al. (1995), except that using fluorescent-labeled primers. Detail procedures is referred to Kim et al. (2009). Four primer combination was used to perform selective amplification (HEX-*Eco*-CAAC/*Mse*-ACGT, HEX-*Eco*-CACT/*Mse*-ACGT, 6-FAM-*Eco*-CATG/*Mse*-ACGT, 6-FAM-*Eco*-CACC/*Mse*-ACGT). The amplified products were separated on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Electropherograms were analyzed using PeakScanner version 1.0 (Applied Biosystems). Binary matrix [presence (1) /absence (0)] of AFLP bands was prepared using RawGeno package v.2.0-1 (Arrigo et al. 2012) implemented in a R package. Scoring parameters is as follows: minimum intensity, 80 rfu; reproducibility limits, 85%; minimum bin width, 1 bp; maximum bin width, 2 bp.

Chloroplast DNA Sequencing

Three cpDNA regions were amplified and sequenced as follows: (1) the *atpI-atpH* region (Tsumura et al. 1996), (2) the *psbM-trnD* regions (Lee and Wen 2004), and (3) the *trnT-L* region (Taberlet et al. 1991; Table S3). To amplify *trn*T-L and *trn*D-*psb*M regions, reaction mixture contained 10 × *Taq* buffer (1.5 mM $MgCl_2$), dNTP (each 0.25 mM), primer (forward and reverse, each 10 pmol), 1 U *Taq* DNA polymerase (Solgent, Korea), and genomic DNA (200 ng in 2 μL). Thermal cycler (PTC-200, MJ Research, USA) was programmed for 3 min at 94°C (pre-denaturation), followed 35 cycles of 1 min at 94°C (denaturation), 1 min at 53°C (annealing), 1 min 30 sec at 72°C and

final stage of 5 min at 72°C.

To amplify the *atp*I-*atp*H region, reaction mixture contained 10 × Excel Speed-*Pfu* Buffer, dNTP mix (each 0.2 mM), primer (forward and reverse, each 10 pmol), 1.25 unit Excel Speed-*Pfu* polymerase (Inclone, Korea) and genomic DNA (200ng in 2 μL). Thermal cycler was programmed for 3 min at 94°C (pre-denaturation), followed 40 cycles of 20 sec at 94°C (denaturation), 40 sec at 57°C (annealing), 2 min 30 sec at 72°C and final stage of 5 min at 72°C.

Amplified DNA products were checked by electrophoresis in 1.5% agarose gel and purified using purification kit (Gel & PCR Purification System, Solgent, Korea). The purified samples were directly sequenced in both directions using the amplification primers on ABI Prism 3730XL DNA sequencer (Applied Biosystems). Complementary DNA sequences were assembled for each accession using Codon code aligner v.5.0.2 (CodonCode Corporation, Dedham, MA, USA) to evaluate chromatograms for confirmation of base calls and to edit contiguous sequences. Multiple sequence alignment was performed ClustalW (Thompson et al. 1994).

AFLP Data Analyses

Binary matrix was converted to input form of many analysis program using AFLPdat (Ehrich 2006) implemented in a R package. Afterward, monomorphic bands across all individuals were excluded from further analysis (Keiper and McConchie 2000). To estimate genetic diversity on the basis of AFLP markers, percentage per loci (*PPL*) and Nei's gene diversity (H_E; Nei 1973) were calculated with POPGENE v.1.31 (Yeh et al. 1997). Genetic differentiation (θ^B) using Bayesian approach were estimated using Hickory v.1.1 (Holsinger and Lewis 2003). To ensure consistency of results (nBurnin = 50,000, nSamples = 250,000, thin = 50 in each run), we used five runs for each of three models (f = full, 0, free). Model selection was based on the deviance information criterion (DIC) (Spiegelhalter et al. 2002). Although model with the smallest DIC is preferred, a difference of > 6 DIC units among models is required in selecting one model over another (Holsinger and Lewis 2005).

To assess the hierarchical genetic structure of a population, we conducted the Analysis of Molecular Variance (AMOVA) analysis using Arlequin v.3.5 (Excoffier and Lischer 2010). Significant levels of variance were obtained from tests including 1000 permutations for each analysis. We divided the 37 populations of *C. japonica* into five regions, depending on the geographical distance of over 100 km and isolation by the sea: (1) mainland of South Korea, (2) Jeju Island, (3) Kyushu, (4) Okinawa, and (5) Taiwan.

To illustrate the genetic relationships between populations, Neighbor-joining (NJ) dendrogram was generated based on Nei's genetic distance (Nei and Li 1979) using MEGA v.6 (Tamura et al. 2013). Correlation analysis between genetic diversity and geographical parameter (latitude) was carried out using R v.3.2.0 (R Core Team 2015). Discriminant Analysis of Principal Components (DAPC) was performed to estimate the number of clusters (K) based on multivariate principal component analysis with a discriminant analysis (Jombart et al. 2010) using R package ADEGENET (Jombart 2008). The optimal number was based on the value of the Bayesian information criterion (BIC). We used 150 principal components (PCs) being able to 80% of total loci in Discriminant Analysis and selected K value associated the lowest BIC before increase again. Scatter plot was generated using first two DA axes enough showing genetic difference between clusters. All of plots was obtained with SigmaPlot v.10.0 (Systat, San Jose, CA, USA).

STRUCTURE v.2.3.4 was used to assign individuals into K groups to infer the number of populations of *C. japonica* based on genetic similarity rather than geographic information using a Markov Chain Monte Carlo (MCMC) algorithm (Falush et al. 2007). STRUCTURE was run by varying the number of clusters (K) from 1 to 10. We used the admixture model assuming no linkage between the loci and performed clustering without a priori information on populations. Five replicates of STRUCTURE were run with 100,000 MCMC steps after a burn-in of 10,000 iterations. We compiled results from our STRUCTURE HARVESTER (Earl and vonHoldt, 2012; available at http://taylor0.biology.ucla.edu/structureHarvester/). To determine the most likely K, we calculated the posterior probabilities of the mean of five runs at each K. The resulting proportions for each individual were used to generate a posterior probability graph showing the species contributions for each individual.

Haplotypes Data Analyses

Using DnaSP v.5 (Librado and Rozas 2009), haplotypes were assigned from the combined three cpDNA dataset. The evolutionary relationships of the inferred cpDNA haplotypes was constructed by coalescent simulation using the median-joining model implemented in Network v.4.6 (http://fluxus-engineering.com). A statistical parsimony haplotype network, based on the matrix of pairwise differences between cpDNA haplotypes, was also obtained with the aid of the TCS v.1.21 (Clement et al. 2000) using 95% connection probability limit and treating gaps as single evolutionary events.

Genetic differentiation parameters (*Gst*, the differentiation based on the frequency of the haplotypes; *Nst*, the similarities between haplotypes) were calculated using PERMUT v.1.0 (http: //www.pierroton.inra.fr/genetics/labo/Software/) with the statistical significance determined by 5,000 replicates. Then for the test of the existence of phylogeographic structure (a situation when closely related haplotypes are more often found in the same area than less closely related haplotypes) (Pons and Petit 1996), we used the U-statistics test.

Molecular Dating Analyses

For the divergence time estimation, we analyzed the *C. japonica* haplotypes within a broad phylogenetic framework of the tribe Theeae to enable the fossil calibrations. We included 18 taxa of *Camellia* and 7 outgroup taxa which were selected based on the result from previous phylogenetic analysis of Theeae (Zhang et al. 2014). Sequences were downloaded from GenBank for all other *Camellia* species and outgroups except the *C. japonica* haplotypes identified in this study. Taxa sampled and GenBank accession numbers for the three data sets are listed in Table S4.

Bayesian dating based on a relaxed-clock model was used to estimate the divergence time of the *C. japonica* haplotypes using BEAST v.1.8.2 (Drummond et al. 2012). BEAST employs a Bayesian Markov chain Monte Carlo (MCMC) approach to co-estimate topology, substitution rates and node ages. Based on the Akaike information criterion (AIC; Akaike 1974), Modeltest v.3.1 (Posada and Crandall 1998) assigned the GTR + I + G model of molecular evolution to the cpDNA combined set. The tree prior model (Yule) was implemented in the analysis, with rate variation across branches assumed to be uncorrelated and lognormally distributed. Posterior distribution of parameters were approximated using two independent MCMC analyses of 10 million generations (sampling once every 1000 generations). Convergence of the stationary distribution was checked by visual inspection of plotted posterior estimates using Tracer v.1.5 (Rambaut and Drummond 2007). After discarding the first 1000 (10%) trees as burn-in, the samples were summarized in the maximum clade credibility tree using TreeAnnotator v.1.6.1 (Drummond and Rambaut 2007) with a posterior probabilities (PP) limit of 0.5 and summarizing mean node heights. Mean and 95% higher posterior densities (HPDs) of age estimates are obtained from the combined outputs using Tracer. The results were visualized using FigTree v.1.3.1 (Rambaut 2009). Two calibration points were used to estimate the divergence time of *C. japonica* populations in East Asia: (1) the stem node of *Camellia* was constrained with a uniform distribution from 18.0 to 23.9 mya (C1 in Fig. 6) based on the fossil of *C.*

japonoxyla, a species from the late Miocene Yanagida Formation of the Noto Peninsula, Japan (Suzuki and Terada 1996) because previous studies have confirmed the systematic position of this fossil and its age (Li et al. 2003; Jeong et al. 2004; Lim et al. 2010); (2) the crown of *Polyspora* was constrained to 7.3–18.7 mya (C2) with a uniform distribution following Zhang et al. (2014) who estimated the divergence time of the tribe Theeae (Theaceae s.s.) using cpDNA sequences.

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Author's Contributions

H-KC designed this research; YR, IRK, MHS studied the literature and collected the specimens, also surveyed the field works; JJ and CK analyzed data and helped YR in writing the manuscript. All authors agreed on the contents of the paper and declared that no competing interests exist.

Supporting Information

Fig. S1. Neighbor-joining dendrogram of 37 populations of *C. japonica* based on Nei's genetic distance.
Fig. S2. Scatterplots of results from discriminant analysis (DA) of principal components (DAPC) of 37 sub-populations of *Camellia japonica.*
Table S1. Estimated posterior probabilities for *K*. Most likely number of genetic clusters (*K*) identified with STRUCTURE is shown in bold.
Table S2. Outgroup probability between haplotypes. Results are based on chloroplast DNA and analyzed by the TCS program.
Table S3. Regions of cpDNA and nine primer pairs used in pre-screening for haplotype analysis.
Table S4. Accession numbers of NCBI used molecular clock analysis in this study.

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