Ann. Bot. Fennici 51: 39–48 ISSN 0003-3847 (print) ISSN 1797-2442 (online)
Helsinki 20 January 2014

Genetic differentiation and introgression among Korean evergreen *Quercus* (Fagaceae) are revealed by microsatellite markers

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Received 20 July 2013, final version received 26 Sep. 2013, accepted 17 Oct. 2013

Lee, J. H., Jin, D. P. & Choi, B. H. 2014: Genetic differentiation and introgression among Korean evergreen *Quercus* (Fagaceae) are revealed by microsatellite markers. — *Ann. Bot. Fennici* 51: 39–48.

Studies of hybridization among interfertile oaks (*Quercus*, Fagaceae) help us understand the interspecific limits and evolutionary implications pertaining to these species. However, in East Asia, little attention has been paid to introgression and hybridization, especially in relation to evergreen oaks. Our objective was to examine the natural hybridization and introgression among related taxa. We performed a genetics analysis of 158 individuals from three evergreen *Quercus* species in Korea (*Q. acuta*, *Q. salicina*, and *Q. glauca*). Focusing on six microsatellite loci, we investigated the typical morphological characteristics of each species. Bayesian admixture analyses and measures of genetic differentiation (F_{ST}, R_{ST}, G_{ST}, and G'_{ST}) were conducted. A feasible level of coincidence was found between the morphological classification and genotype of each individual, and the differentiation was significant between all pairings. However, six individuals showed mixed genotypes with other clusters, implying that they are putative hybrids. Our results suggest that these mixed genotypes among morphologically pure individuals might have arisen from backcrosses between a hybrid and one of the parental species.

Introduction

The natural hybridization that is found in many plant groups has important evolutionary implications (Rieseberg & Carney 1998). Because the genus *Quercus* (Fagaceae) is interfertile (Cottam *et al*. 1982), it has served as a model for studying evolutionary processes and speciation (Neophytou *et al*. 2010). Interspecific introgression within *Quercus* can have significant ecological consequences, enabling colonization of novel habitats (Petit *et al*. 2003) and influencing plant responses to various selective pressures in the environment (Dodd & Afzal-Rafii 2004).

Because the number of reported cases of hybridization and introgression has increased, gene flow among *Quercus* species has become a widely accepted and broadly studied phenomenon, particularly in Europe and North America (e.g., Craft *et al*. 2002, de Casas *et al*. 2007, Valbuena-Carabaña *et al*. 2007, Ortego & Bonal 2010, Moran *et al*. 2012). However, in East Asia, little attention has been paid to this issue in *Quercus*. Moreover, few interspecific studies

of any deciduous species have been carried out (e.g., Ishida *et al*. 2003, Matsumoto *et al*. 2009).

Quercus acuta is endemic to Korea and Japan, and is a major component of temperate evergreen broad-leaved forests (Ohashi *et al*. 2006, Lee & Choi 2010). It is widely distributed, frequently overlapping areas populated with *Q. salicina* in Korea. Both also co-occur with *Q. glauca* on several islands. These evergreen trees follow an ecological and elevational gradient, with *Q. acuta* growing at the highest elevations (average: 725 m a.s.l.), followed by *Q. salicina* (Okano & Suzaki 1989, Ito *et al*. 2007). By contrast, *Q. glauca* has a low-elevational range (average: 255 m a.s.l.), generally forming a cluster with pioneer and/or deciduous species (Ito *et al*. 2007). The environmental requirements of *Q. glauca* appear to contrast with those for *Q. acuta*.

Although morphological traits can be a tool for identifying suspected hybrids in oaks (Neophytou *et al*. 2007), they do not allow for inferences regarding ancient introgression. Furthermore, their use in such diagnoses of hybridization is limited when applied to the evergreen oaks that are the focus of our research. Each species tends to show a high degree of morphological uniformity (Chang 2007, Lee 2012). A few morphological hybrids between them have been described, including one record of a pairing between *Q. acuta* and *Q. glauca* in Japan (Makino 1941). Thus, their genetic markers can provide additional insight into the patterns of gene exchange for hybridization and introgression. A population genetic study using ISSR and RAPD markers did not reveal any evidence of hybridization in the Korean evergreen oaks (Choi 2001). However, the results from that investigation had only limited capacity to detect such hybrids because the use of dominant markers did not accommodate the scoring of genotypes. In contrast, other research findings have suggested that the interspecific gene flow between them appears to indicate the sharing of cpDNA haplotypes in Korea and Japan (Aoki *et al*. 2003, Lee *et al*. 2011). Because of this, alternative, powerful molecular tools must be used to elucidate the genetic evidence for this hybridization and introgression.

Microsatellites demonstrate biparental inheritance, because both parental species contribute alleles. Moreover, high allelic diversity enables one to identify F_1 and even backcrossed hybrids if the parental species are sufficiently differentiated (Abraham *et al*. 2011, Yun *et al*. 2011). This technique has already aided researchers in distinguishing hybrids between two morphologically pure Mediterranean evergreen oaks, *Q. coccifera* and *Q. ilex* (Ortego & Bonal 2010). Thus, microsatellite markers can be a complementary and reliable molecular means for studying hybrid complexes (e.g., Craft *et al*. 2002, Burgarella *et al*. 2009, Lumaret & Jabbour-Zahab 2009, Neophytou *et al*. 2010, Abraham *et al*. 2011, Moran *et al*. 2012).

Here, we used such markers to capture genetic imprints for interspecific hybridization among *Quercus* species sampled from three different regions in Korea. We tested whether interspecific hybrids could be detected in morphologically distinct individuals of *Q. acuta* and related taxa (*Q. salicina* and *Q. glauca*).

Material and methods

Sampling and plant material

We collected 158 adult individuals of *Quercus acuta* (67), *Q. salicina* (62), and *Q. glauca* (29) from three sites along the Korean Peninsula (Fig. 1). These selections were made after considering their distribution in the southern coastal areas, and included the islands of Wan, Geumo, and Jeju. For *Q. glauca*, only one population was sampled from Jeju because its occurrence is infrequent in this region. We also collected one putative morphological hybrid between *Q. acuta* and *Q. salicina* on Wan. Voucher specimens were deposited in the herbarium at Inha University, Incheon.

These three species had marked morphological differences (Chang 2007, Lee 2012). For example, the leaves of *Q. acuta* had entire margins and were pale-green and glabrous on their undersides. Those of *Q. salicina* had sharp teeth on the upper half of the margins, and were waxy-white and glabrous underneath, while the leaves of *Q. glauca* had subacute teeth on the upper half of the margins, but had a yellowish-brown pubescence on the underside. Two types

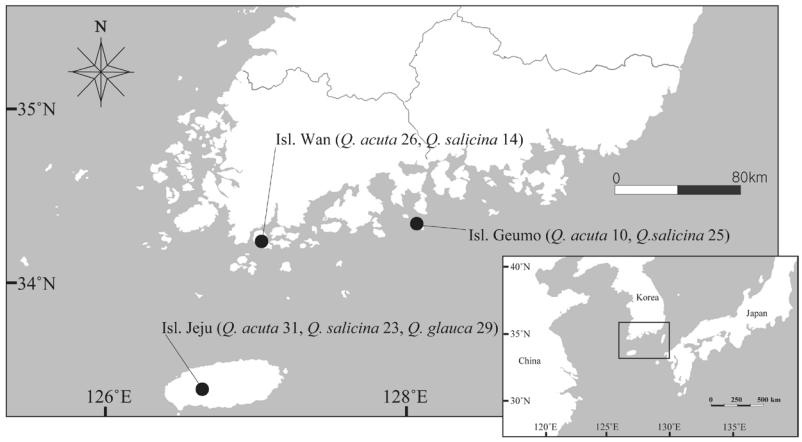

Fig. 1. Sampling sites. Values in parentheses after each species refer to number of collected individuals.

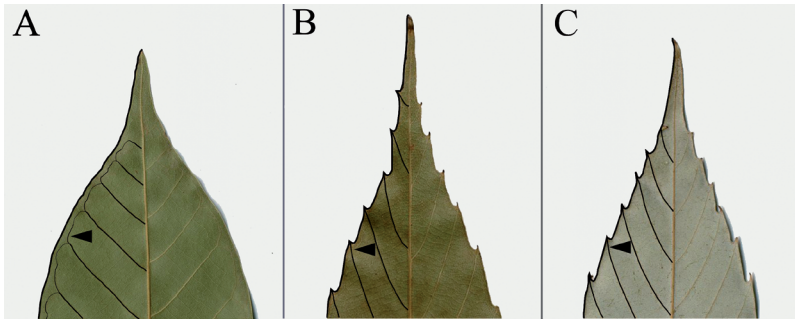

Fig. 2. Lower surface of leaf of (**A**) *Quercus acuta*, (**B**) putative hybrid, and (**C**) *Q. salicina*. Secondary veins and leaf margins are traced on left sides. Arrows show the shape of secondary vein.

of venation were apparent: (1) craspedodromous for *Q. glauca* and *Q. salicina*, where the secondary veins stretched up to the margin, and (2) eucamptodromous for *Q. acuta*, with secondary veins extending close to the margin, but anastomosing before they intersected there (Fig. 2). This feature enabled us to identify it and separate it from the other species in our identifications. A possibly hybrid individual, manifesting an intermediate form and unique diagnostic traits, was also added to our STRUCTURE analysis.

Microsatellite genotyping

All individuals were genotyped at six nuclear microsatellite loci: MSQa1, MSQa4, MSQa6, MSQa10, MSQa13, and MSQa14 (Lee *et al.* 2010). We also used previously obtained, partially genotyped data (unpublished) for *Q. acuta* at five loci: MSQa4, MSQa6, MSQa10, MSQa13, and MSQa14. Genomic DNA was extracted from fresh or silica gel-dried leaves, using a G-spin™ IIp kit for plants (iNtRON, Inc., Seongnam, Korea). PCR was conducted with a GeneAmp® PCR System 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The M13(-21) (5´-TGTAAAACGACGGCCAGT-3´) sequence tag method was used to label the 6 primers (Schuelke 2000). Each reaction mixture contained 200 μM dNTPs (GeneCraft, Lüdinghausen, Germany), 1 × PCR buffer with 1.5 mM $MgCl_2$, 1 U of Taq DNA polymerase (TaKaRa, Inc., Seoul, Korea), 10 ng of DNA, and an appropriate concentration of primers in a total volume of 30 μl. The mixture also contained a forward M13(-21)-tagged primer (0.08 μM), reverse primer (0.3 μM), and M13(-21)-labeled 6-FAM fluorescent dyes (0.3 μM). Conditions included initial denaturation at 94 °C for 2 min, followed by 38 cycles at 95 °C for 30 s, 52 to 56 °C for 45 s (the annealing temperature depending upon locus; *see* Lee *et al.* 2010), and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Fluorescently labeled PCR products were electrophoresed con-

currently with the GeneScan™-500LIZ™ Size Standard on an ABI 3730xl sequencer. Sizes were determined with GENEMAPPER ver. 3.7 (Applied Biosystems).

Data analysis

We used GenAlex ver. 6.3 (Peakall & Smouse 2006) to calculate standard genetic diversity parameters, i.e., allele frequencies, observed heterozygosities (H_o), and expected heterozygosities (H_e). With the same software, genetic differentiation was determined between species, including computations of F_{ST} (Wright 1951) and R_{ST}, an indication of *R*-statistics (Slatkin 1995), similar measures developed specifically for microsatellite by assuming the generalized stepwise mutation. Values of R_{ST} were calculated to avoid biased estimations, when using only F_{ST}. The statistical significance at each locus and multilocus was tested using permutation with 999 steps. Subsequently, their relative differentiation, G_{ST} (Nei 1973) and standardized differentiation statistic, G'_{ST} (Hedrick 2005) were calculated using Software for Measurement of Genetic Diversity (SMOGD; Crawford 2010). The latter values were estimated at the 95% confidence interval through a bootstrap procedure (500 steps). To assess the diagnostic power of each marker, we also estimated δ, the allele frequency differential among species (Shriver *et al*. 1997). All analyses were performed only with putative purebred individuals that had been selected after a preliminary screening for potential hybrids (*see* below).

To evaluate species assignments and hybridization, we used a Bayesian model-based clustering method (STRUCTURE 2.0; Pritchard *et al*. 2000). This enabled us to assign individuals to *K* populations (= species in this study), based on multilocus genotype data. We employed the admixture model with correlated allele frequencies and also applied STRUCTURE for all samples, including the one considered a morphological intermediate. Ten runs were conducted, which allowed us to determine how well the genetic structure within the entire data set corresponded to phenotypic assignments. Data were examined for $K = 1, \ldots, 7$ to estimate the true number of clusters. This process involved a burn-in period of 10^5 iterations with data collected for 10^6 MCMC cycle iterations. The most appropriate number of clusters (K) was estimated based on the Evanno method (Evanno *et al*. 2005) as implemented in STRUCTURE HARVESTER (Earl & von Holdt 2012).

The posterior probabilities (q) of STRUCTURE provide the estimated proportion of an individual's genome that originates from each cluster. Because each individual was assigned to a cluster based on its threshold value (T_q), determining the appropriate T_q was important. Therefore, we produced artificial genotype data for purebred and hybrid samples that were created with HYBRIDLAB ver. 1.1 (Nielsen *et al*. 2006) according to the procedure of Vähä and Primmer (2006). First, we selected individuals (*Q. acuta*, $n = 63$, *Q. salicina*, $n = 61$, *Q. glauca*, $n = 28$) that had T_q values > 0.90 for the major cluster. Then, based on estimated allele frequencies from selected individuals, we generated 1000 purebreds for each species plus 40 F_1 hybrids and 80 backcrosses per pair via HYBRIDLAB. These genotype data were simulated with STRUCTURE. Finally, we assessed the efficiency, accuracy, and performance in simulated results based on each T_q (0.75, 0.80, 0.85, and 0.90). To do so, we set the highest efficiency for identifying a purebred at $T_q = 0.75$. However, for identifying hybrids (F_1 hybrid or backcross), $T_q = 0.90$ showed the highest efficiency and accuracy. Total performance for distinguishing purebreds and hybrids was also highest at $T_q = 0.90$, so that value was used for T_q. In classifying each individual as a purebred ($q \geq T_q$) or hybrid ($q < T_q$), we considered cases where an individual had a q value < T_q for one cluster but $q < 1 - T_q$ for each of the other two clusters. For these, we concluded that the majority of its genome came from within one species and was not significantly influenced by the other species. Thus, it could also be classified as a pure species (e.g. Lepais *et al*. 2009).

Results

All six microsatellites were highly variable across species, presenting three to 20 alleles per locus. In particular, the allelic diversity of

Table 1. Genetic characteristics of six microsatellite loci for three *Quercus* species.

Locus	*Q. acuta*					*Q. salicina*					*Q. glauca*				
	N_a	N_p	H_o	H_e	range	N_a	N_p	H_o	H_e	range	N_a	N_p	H_o	H_e	range
MSQa1	4	1	0.222	0.240	273–288	5	1	0.689	0.663	270–285	6	1	0.714	0.649	273–291
MSQa4	8	1	0.683	0.811	295–317	9	2	0.377	0.711	301–319	9	0	0.250	0.700	295–319
MSQa6	20	9	0.810	0.846	312–362	3	1	0.426	0.369	308–332	18	6	0.964	0.914	296–364
MSQa10	4	1	0.333	0.396	331–339	7	3	0.459	0.637	323–341	9	6	0.857	0.736	335–353
MSQa13	13	4	0.825	0.886	294–322	13	2	0.820	0.838	288–314	9	1	0.893	0.862	290–316
MSQa14	13	0	0.571	0.638	256–282	12	1	0.754	0.846	256–288	14	3	0.893	0.892	256–292

N_a = number of alleles, N_p = unique alleles, H_o = observed heterozygosity, H_e = expected heterozygosity.

MSQa6 was very different for each species, i.e., 20 alleles revealed in *Q. acuta* versus only three in *Q. salicina*. Although the three species had alleles in common at all loci, about half or more of the alleles at four loci, excluding MSQa13 and MSQa14, were not shared. Each species also had 10 to 17 unique alleles. The genetic parameters for each species and marker are presented in Table 1. Most of the loci had high discriminatory power, with the allele frequency differential ranged from $\delta = 0.359$ to $\delta = 0.959$, based on pairs and each locus (Table 2).

According to the program STRUCTURE, the most likely K (i.e., the most appropriate number of clusters based on multilocus genotype data) is the one for which $\ln\Pr(X|K)$ is the highest. Here, the likelihood for partitioning of the data increased sharply from $K = 1$ to $K = 3$ before either slightly increasing or decreasing from $K = 3$ to $K = 7$. The optimal value of ΔK was obtained at $K = 3$ (Fig. 3). These results implied that the uppermost level of the structure was at $K = 3$, which provided a relatively meaningful explanation corresponding to the species. That is, these three well-defined groups were congruent with their observed phenotypes (Fig. 4). Therefore, we used $K = 3$ in our analysis.

Using STRUCTURE, we detected six putative hybrids with a threshold $T_q = 0.90$. The other individuals had a very high probability of belonging to a pure species. Four of the putative hybrids were identified according to morphological criteria as being *Q. acuta*, one as *Q. glauca*, and one as *Q. salicina*. In addition, one individual (Voucher no. *16160*) was considered

Table 2. Allele frequency differential (δ) among species at six microsatellite loci.

Locus	Species pairs		
	Q. acuta vs. Q. salicina	*Q. acuta vs. Q. glauca*	*Q. salicina vs. Q. glauca*
MSQa1	0.873	0.855	0.359
MSQa4	0.687	0.565	0.878
MSQa6	0.959	0.673	0.929
MSQa10	0.460	0.683	0.670
MSQa13	0.593	0.520	0.510
MSQa14	0.515	0.464	0.373
Mean	0.681	0.627	0.620

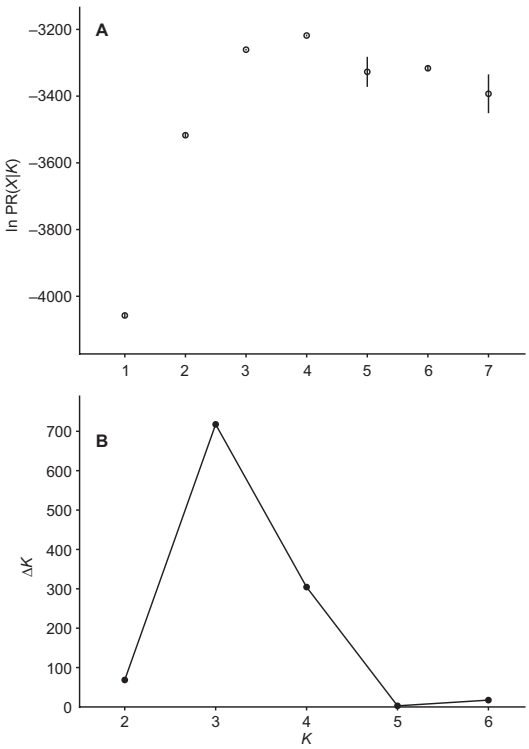

Fig. 3. Summary of runs for *K* values ranging from 1 to 7 by STRUCTURE. — **A**: Mean lnPr(*X*|*K*) ± SD for each *K* plotted against *K*. — **B**: Δ*K* plotted against *K*.

morphologically intermediate, suggesting the possibility that it was a hybrid between *Q. acuta* and *Q. salicina* (Table 3 and Fig. 2).

Estimated genetic differentiation between species (F_{ST}) was significant for each locus and multilocus estimate for all three comparisons ($p < 0.001$; Table 4). Although R_{ST} was not significant at all loci, it was significant for multilocus estimates. Values for F_{ST} and the other statistical measures demonstrated that this differentiation was strongest between *Q. acuta* and *Q. salicina* ($F_{ST} = 0.246$, $R_{ST} = 0.524$, $G_{ST} = 0.151$, and $G'_{ST} = 0.686$). For the other two pairs, values for F_{ST} and G_{ST} were higher between *Q. acuta* and *Q. glauca* than between *Q. glauca* and *Q. salicina*. By contrast, R_{ST} and G'_{ST} were higher between *Q. glauca* and *Q. salicina*. This indicates that the degree of differentiation was similar between *Q. acuta* and *Q. glauca*, as well as between *Q. glauca* and *Q. salicina*.

Discussion

STRUCTURE clustering analysis has frequently been used to diagnose hybrids and admixed individuals. This method has performed well in previous studies, particularly when the markers present a high degree of divergence in the parental species (e.g., Craft *et al*. 2002, Burgarella *et al*. 2009). Our analysis assigned nearly all individuals with a high probability ($q > 0.90$) to one of three genetically defined clusters, resulting in an almost perfect match with previously classified morphotypes. However, six individuals showed mixed genotypes with other clusters, suggesting that they are putative hybrids (i.e., individuals with assignments split between both clusters: $0.10 < q < 0.90$). Our findings support earlier hypotheses that haplotype sharing between species could indicate past hybridization and introgression (Aoki *et al*. 2003, Lee *et al*. 2011). The most plausible explanation for this existence of mixed genotypes among morphologically pure individuals might be that they came from backcrosses between a hybrid and one of the parental species. This may have resulted in long-lasting introgression, as has been suggested for Mediterranean

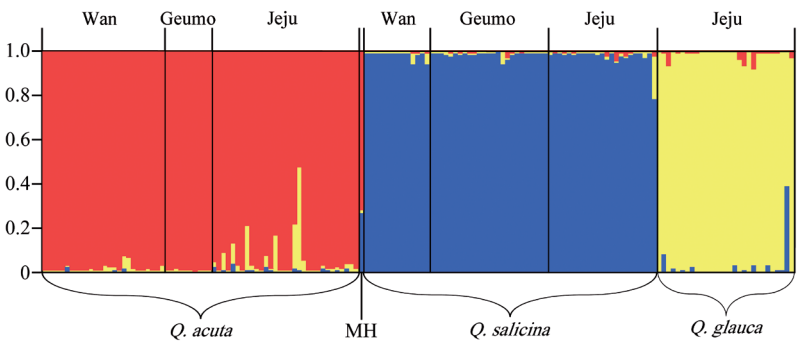

Fig. 4. Genetic assignments based on Bayesian method, with *K* = 3 corresponding to morphological appearance, as conducted by STRUCTURE. Each individual is represented by single vertical line and assigned to genetic cluster. Morphologically intermediate 'MH' individual from Wan was included.

evergreen oaks (Burgarella *et al*. 2009, Lumaret & Jabbour-Zahab 2009, Ortego & Bonal 2010).

For each paring, we found that interscecific gene exchange was more frequent between *Q. acuta* and *Q. glauca*. In an associated phenomenon, numerous studies have examined hybridization and introgression in closely related oaks that show high levels of such an exchange (Heuertz *et al*. 2006, Valbuena-Carabaña *et al*. 2007, Ortego & Bonal 2010, Moran *et al*. 2012). Interspecies gene flow is expected to be lower between more distantly related species (Lumaret & Jabbour-Zahab 2009). Therefore, we may presume that *Q. acuta* is more phylogenetically closely related to *Q. glauca* than to *Q. salicina*, although it was difficult here to elucidate such relationships and close degrees directly. This finding was robustly supported by our data that revealed only limited introgression between *Q. acuta* and *Q. salicina*, two species with a high degree of differentiation. By comparison, the genetic evidence was strong for hybridization and introgression between *Q. acuta* and *Q. glauca*. Thus, we may infer that such a phylogenetic pattern would be strongly indicative of a significant presence by hybridization and introgression. However, we detected very low levels of admixture between *Q. acuta* and *Q. glauca* in our Bayesian clustering analysis, and their significant differentiation ($p < 0.001$) could be a sign that hybridization occurred at a low rate.

Quercus acuta trees may be able to maintain their morphological and genetic identity despite introgression from *Q. glauca*. This arises because of ecological sorting in both species, with *Q. acuta* being distributed mainly on rich soils at the highest elevations (Obata 1936), i.e., sites where *Q. glauca* trees do not develop opti-

Table 3. Posterior probabilities for putative hybrids after STRUCTURE analyses.

Individual voucher no.	Sampling site	MSA	Inferred cluster		
			Q. acuta	*Q. salicina*	*Q. glauca*
912558	Jeju	*Q. acuta*^{QAG}	0.791	0.009	0.200
912542	Jeju	*Q. acuta*^{QAG}	0.832	0.005	0.163
912547	Jeju	*Q. acuta*^{QAG}	0.783	0.016	0.200
910019	Jeju	*Q. acuta*^{QAG}	0.524	0.011	0.465
912038	Jeju	*Q. salicina*^{QGS}	0.026	0.782	0.192
912089	Jeju	*Q. glauca*^{QGS}	0.005	0.392	0.603
16160	Wan	Intermediate^{QAS}	0.721	0.270	0.008

MSA indicates species assigned to *Q. acuta*, *Q. salicina*, *Q. glauca*, or intermediate based on morphological characteristics. Posterior probabilities represent the proportion of the genotype from its origin. QAG = putative hybrid between *Q. acuta* and *Q. glauca*, QAS = putative hybrid between *Q. acuta* and *Q. salicina*, QGS = putative hybrid between *Q. salicina* and *Q. glauca*.

Table 4. Measures of genetic differentiation among three *Quercus* species.

Locus	*Q. acuta vs. Q. salicina*				*Q. acuta vs. Q. glauca*				*Q. glauca vs. Q. salicina*			
	F_{ST}	R_{ST}	G_{ST}	G'_{ST}	F_{ST}	R_{ST}	G_{ST}	G'_{ST}	F_{ST}	R_{ST}	G_{ST}	G'_{ST}
MSQa1	0.536*	0.498*	0.364	0.974	0.579*	0.593*	0.361	0.952	0.084*	0	0.044	0.218
MSQa4	0.186*	0.426*	0.104	0.786	0.101*	0.072	0.057	0.426	0.267*	0.689*	0.157	0.947
MSQa6	0.385*	0.645*	0.240	0.996	0.061*	0.268*	0.031	0.543	0.362*	0.276*	0.192	0.903
MSQa10	0.164*	0.091*	0.090	0.285	0.296*	0.528*	0.156	0.576	0.187*	0.498*	0.101	0.565
MSQa13	0.083*	0.306	0.043	0.617	0.036*	0.173	0.019	0.311	0.081*	0.060	0.042	0.565
MSQa14	0.123*	0.061	0.066	0.459	0.092*	0.012	0.045	0.357	0.029*	0	0.015	0.241
Mean	0.246*	0.524*	0.151	0.686	0.194*	0.242*	0.112	0.528	0.168*	0.292*	0.092	0.573

* indicates significant differentiation ($p < 0.001$).

mal populations (Ito *et al*. 2007). By comparison, we noted that hybrids between *Q*. *acuta* and *Q*. *glauca* exhibited just one original parental morphotype as *Q*. *acuta*, which eventually showed a predominant direction of gene flow from *Q*. *glauca* toward *Q*. *acuta*. Although the sources of this unidirectional tendency remain uncertain, we can speculate on the extent to which a few of the many possible factors may have contributed. Biases in the relative abundance of parental species can influence flow directions and hybridization rates (Lepais *et al*. 2009). However, it is unlikely that *Q*. *glauca* was more dominant than *Q*. *acuta* on Jeju (personal observation). Alternatively, the evidence put forward for such a tendency in gene flow between the two species on Jeju is the past extinction of *Q*. *glauca* in Korea. Its recent migration is obvious, as suggested by Huang *et al*. (2002). Conversely, *Q*. *acuta* has survived long-term along the Korean Peninsula, showing a pattern that parallels that of the evergreen *Neolitsea sericea*, which has a similar distribution range there (Lee *et al*. 2013). Thus, we suggest that the genome for *Q*. *acuta* has been maintained despite this invasion from *Q*. *glauca*, possibly because the former populates a relatively larger and more stable habitat on Jeju than in the other regions examined here.

Genotypes and morphotypes were perfectly matched for *Q*. *acuta* and *Q*. *salicina*, as shown by our Bayesian analysis. All measures of genetic differentiation applied here revealed the highest values between those two species. Moreover, the "morphologically intermediate" tree that we found growing within the dominance range between the two on Wan indicated a mixed genotype. The leaf morphology of this intermediate tree was similar in color to *Q*. *acuta* on the lower surface, but similar to *Q*. *salicina* with regard to venation and marginal teeth. Our intermediate sample had pale-green, serrate leaves that were glabrous and craspedodromous in venation (Fig. 2). In a study of *Fraxinus* species, Fernandez-Manjarres *et al*. (2006) suggested that first-generation and/or sterile hybrids can be distinguished by their intermediate morphological characters. Perhaps our morphologically intermediate specimen, considered an F_1 hybrid between *Q*. *acuta* and *Q*. *salicina*, is sterile, so that back-crossing is not possible.

In conclusion, we detected, albeit rare, hybrids in apparently pure individuals among *Q*. *acuta*, *Q*. *glauca* and *Q*. *salicina*. Their genetic differentiation is significant. This infrequent hybridization is to be expected in distantly related species. We cannot fully explain the cause of unidirectional gene flow from *Q*. *glauca* toward *Q*. *acuta*. Therefore, our findings open new questions that must be addressed. Future experiments should involve examining large-scale patterns of morphology, genetic structure, and ecological correlates of *Q*. *acuta* and *Q*. *glauca*, with this research including samples from Japan as well.

Acknowledgments

We thank our colleagues J. Y. Lee, W. B. Cho, D. H. Lee, and I. S. Choi, at the Plant Systematics Laboratory of Inha University for providing help with our statistical analysis and commenting on the manuscript. This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (no. 2012R1A2A2A01011428).

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