

**DIFFERENTIATION AND HYBRIDIZATION BETWEEN
QUERCUS CRISPULA AND *Q. DENTATA* (FAGACEAE):
INSIGHTS FROM MORPHOLOGICAL TRAITS, AMPLIFIED
FRAGMENT LENGTH POLYMORPHISM MARKERS, AND
LEAFMINER COMPOSITION¹**

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Quercus crispula and *Q. dentata* (Fagaceae) are dominant members of cool-temperate forests of Japan and are assumed to hybridize in nature. To characterize and discriminate these two species and their hybrids, we carried out multivariate analysis using several morphological traits and principal coordinate analysis using molecular (amplified fragment length polymorphism [AFLP]) data. Further, we examined the composition of *Phyllonorycter* species (leafmining insects) on individuals from a mixed forest. Morphological traits and *Phyllonorycter* composition differ enough in these two oak species to be useful for identification of species and hybrids. AFLP data, however, are less informative because the degree of molecular differentiation between the two species is low. Nine out of 105 individuals from a mixed stand had intermediate morphologies according to the multivariate analysis, and eight out of the nine individuals had intermediate *Phyllonorycter* composition in either one or both of the two study years. These eight individuals were tentatively assigned as hybrids or backcross individuals, and the remaining individual with intermediate morphologies was assigned as *Q. dentata* according to its *Phyllonorycter* composition and the AFLP analysis.

Key words: AFLP; Fagaceae; hybridization; morphological traits; *Phyllonorycter*; *Quercus crispula*; *Quercus dentata*.

The genus *Quercus* (Fagaceae) shows complex patterns of genetic and morphological variation at the inter- and intraspecific levels and has played an important role in the development of concepts of species, speciation, and evolution (Anderson, 1949; Burger, 1975; Van Valen, 1976; Rieseberg and Wendel, 1993). Previous studies on *Q. robur-Q. petraea-Q. pubescens*, *Q. rubra-Q. ellipsoidalis*, and *Q. gambelii-Q. grisea* complexes have indicated that sibling pairs are more distinctly discriminated by morphological or ecological (i.e., adaptive) traits than by isozyme or DNA markers (Jensen et al., 1993; Kleinschmit et al., 1995; Howard et al., 1997; Bruschi et al., 2000; Tomlinson et al., 2000). Morphological or adaptive traits might have differentiated faster than isozymes or DNA markers. In addition, isozymes or DNA markers, which are probably not affected by natural selection, might have been transferred from species to species through hybridization, while alleles responsible for differential adaptation might not have been transferred despite hybridization (Wu, 2001). To address these issues, information on the extent of morphological, ecological, and genetic differentiation and the frequency of hybridization is important, but still insufficient. This is partly because even identification of hybrid individuals of oaks is not simple (Jensen et al., 1993; Kleinschmit et al., 1995; Bruschi et al., 2000; Tomlinson et al., 2000).

In this paper, we investigate differentiation and hybridiza-

tion between *Q. crispula* Blume and *Q. dentata* Thunberg on the basis of morphological traits, amplified fragment length polymorphism (AFLP) markers, and leafminer (*Phyllonorycter*; Gracillariidae; Lepidoptera) composition. These two oaks are widely distributed in central and northern Japan and often co-occur. They are normally discriminated by the presence or absence of stellate hairs on the lower surface of leaf and characteristics of the acorn cap (Ooba, 1989). These two species also differ in some other traits such as leaf thickness and number of lobes of leaf (Ooba, 1989). However, continuous variation is observed in these traits, especially among individuals from mixed stands (Miyazaki, 1989; Ooba, 1989; Hashizume et al., 1994). In addition, there are individuals that are *Q. crispula*-type in some traits and *Q. dentata*-type in other traits. These situations, probably due to hybridization and introgression, often prevent the identification of species and hybrid individuals. In the present study, therefore, morphological data were subjected to multivariate analysis to identify linear combinations of variables that best discriminate between the species studied.

For discrimination of oak species, molecular markers such as isoenzymes, microsatellite DNA, and randomly amplified polymorphic DNA (RAPD) have also been used (Kleinschmit et al., 1995; Samuel et al., 1995; Bondénès et al., 1997; Dumolin-Lapègue et al., 1997; Bruschi et al., 2000; Tomlinson et al., 2000), but no diagnostic markers have been obtained except for a set of RAPD markers that distinguish between *Q. gambelii* and *Q. grisea* from North America (Howard et al., 1997). The AFLP technique used in this study yields a large number of stable markers with which multivariate analysis can be performed. Previous studies using this technique have successfully analyzed genetic diversity and identified closely related species and their hybrids in a number of plants and an-

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imals (Hill et al., 1996; Lu et al., 1996; Sharma et al., 1996; Ajmonemarsan et al., 1997; O'Hanlon et al., 1999; Cresswell et al., 2001; Young et al., 2001), but it has not yet been applied in oak taxonomy.

The patterns of herbivore attack of hybrid plants are variable (Fritz, 1999; Orians, 2000), most likely due to variation in the mode of inheritance of chemical traits that affect herbivore behaviors and performance (Orians, 2000). If parental species have species-specific herbivores and if attractants to these herbivores show codominant or intermediate inheritance, hybrid individuals may harbor herbivores of both parental species. In such case, herbivore composition can be used as a surrogate for detection of hybrid individuals. It has been reported that the present two oak species harbor different *Phyllonorycter* species (Sato, 1991; Fujihara et al., 2001). Here we examined *Phyllonorycter* composition on oak individuals from a mixed stand and ascertained its usefulness in the detection of hybrid individuals.

MATERIALS AND METHODS

Study sites and sampling—The main study area was a belt-shaped forest (about 500 m in width and about 20 km in length) along the Ishikari Coast (43°12' N, 141°19' E) in Hokkaido, northern Japan. In this forest, nearly pure stands of *Q. dentata* develop at the seaside, while mixed stands of *Q. crispula* and *Q. dentata* develop in the inner areas. Trees in this area, especially near the forest edge at the seashore, are dwarfed, probably because of winds from the sea.

Collections of leaves were made from 96 individuals that are found along a transect (about 500 m) across this Ishikari forest from the coastal side to inland. In addition, leaves were collected from nine individuals having intermediate appearance between *Q. crispula* and *Q. dentata*. These nine trees grow at distances of 5–50 m from the transect.

As references, leaves were also collected from pure populations of *Q. crispula* in Hamamasu and Toishiyama and a pure population of *Q. dentata* in Nakaotofuke. Hamamasu (43°00' N, 141°17' E) is located about 40 km north of Ishikari; Toishiyama (43°35' N, 141°28' E) is located about 40 km south of Ishikari; and Nakaotofuke (43°07' N, 143°05' E) is situated about 300 km east of Ishikari. The forests in Hamamasu and Toishiyama are dominated by broad-leaved trees such as *Q. crispula*, *Acer mono*, and *Betula platyphylla*, while the forest in Nakaotofuke is a windbreak dominated by *Q. dentata*. Leaves were collected from 50 oak trees at each location.

Morphological data—From each of 255 trees, 10 shade leaves were collected in mid-summer 1999 and measured for area, perimeter, length, width, number of lobes, and dry mass (i.e., mass after dehydration at 60°C for 48 h). The first two traits (area and perimeter) were measured using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>) after leaf shape was scanned by an image scanner (JX-270, Sharp, Tokyo, Japan). In addition, the density and length of stellate hairs and the presence or absence of solitary and short hairs (Hardin, 1975; Ooba, 1989; Kim et al., 1992) on the lower surface were determined for three leaves from each tree. The measurements were made on a piece (3.46 mm²) that was punched off from the intervein area near the base of each leaf. Analyses were made using mean values of 10 or three leaves.

AFLP data—The AFLP data were collected only for individuals from the mixed stand at Ishikari. Winter buds were collected from 104 out of 105 trees at Ishikari in autumn 1999 and stored at –70°C (buds could not be obtained from one individual). Buds (100 mg) were ground in liquid nitrogen, and total genomic DNA was extracted with QIAquick DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA) according to the supplier's instruction. The DNA extracts (about 1.5 µg from each tree) were stored in 70% ethanol until further manipulations.

The AFLP Ligation and Preselective Amplification Module and *EcoRI/MseI* primers were purchased from PE Applied Biosystems (Foster City, California, USA). The extensions of the primer pairs used in this study are *EcoRI-ACA/MseI-CAG*, *EcoRI-AAG/MseI-CAC*, and *EcoRI-AAC/MseI-CTG*. Digestion and amplification of sample DNA were performed according to the supplier's instruction, and amplified products were electrophoresed on an ABI 373A automated sequencer (PE Applied Biosystems).

The relative mobility of fragments was calculated by the inclusion of an internal size standard within each sample. Digital profiles were visualized with the aid of ABI GENESCAN software (PE Applied Biosystems). The consistency of amplification was ascertained using DNA samples from two or three different buds on each of five trees; fragments that were not observed in all the samples from the same trees were assumed to be unstable and were not scored.

Phyllonorycter composition—Nine species of *Phyllonorycter* (leafminers) were observed on oaks in the study area; seven species have been reported to be specific to *Q. crispula* [*P. acutissimae* (Kumata), *P. similis* Kumata, *P. crenata* (Kumata), *P. pseudolautella* (Kumata), *P. pygmaea* (Kumata) and *P. mongolicae* (Kumata) and *P. matsudai* Kumata] and two to *Q. dentata* [*P. persimilis* Fujihara, Sato and Kumata and *P. leucocorona* (Kumata)] (Sato, 1991; Shibata et al., 2001). They are discriminated to species by the pupal exuviae (Sato, 1991; Fujihara et al., 2001).

The *Phyllonorycter* composition was examined only for individuals from the mixed stand at Ishikari. In the pure populations at Nakaotofuke, Toishiyama, and Hamamasu, the density of *Phyllonorycter* species was low, and it was also difficult to collect a large number of leaves because they were located at 20–30 m in height. In 2000, 61–476 leaves were collected from each of 104 oak trees at Ishikari in late October. In 2001, 123–259 leaves were collected from each of 36 selected trees at Ishikari in mid-October: 15 trees had morphological characteristics of *Q. dentata*, 13 had those of *Q. crispula*, and eight had intermediate morphology (see Results). These leaves were stored outdoors until almost all of *Phyllonorycter* larvae grew to pupae. *Phyllonorycter* pupae were then collected from these leaves and identified to species. Individuals remaining as larvae were reared in plastic cases until pupation.

Analysis—The morphological characteristics of individuals from the mixed Ishikari stand were examined with reference to morphology of pure populations at Nakaotofuke (*Q. dentata*), Toishiyama, and Hamamasu (*Q. crispula*). First, we examined whether each morphological trait significantly differed between *Q. dentata* and *Q. crispula* from the pure populations. In this analysis, ANOVA was applied for traits that showed normal distribution according to a Kolmogorov-Smirnov test ($P > 0.05$); a Kruskal-Wallis test was applied for traits that did not show normal distribution; and a χ^2 test was made for contingency data. The traits that showed significant difference between the two species were then applied to factor analysis to examine multicollinearity, which may bring confusion in discriminant analysis, and traits that varied independently were selected. Then, using the selected traits, canonical discriminant analysis was made on individuals from the three pure populations. A discriminant formula obtained with the above analysis was then used to calculate canonical variate (CV) scores for individuals from the mixed stand at Ishikari. All the statistical analyses except for the Kolmogorov-Smirnov test were performed with JMP 4.0 (SAS Institute, Cary, North Carolina, USA); the Kolmogorov-Smirnov test was made with SPSS 6.1 (SPSS, Chicago, Illinois, USA).

On AFLP data, we performed principal coordinate analysis (PCOA) to assess genetic differentiation between these two oak species. A PCOA allows the assessment of the dimensionality of the data and a description of the major patterns of variation within and between populations (Cresswell et al., 2001). The similarity between each pair of trees was estimated using the Dice's ecological similarity index, S_{ij} (Dice, 1945): $S_{ij} = 2N_{ij}/(N_i + N_j)$, where N_{ij} is the number of markers shared by plants i and j , N_i is the number of markers found in plant i , and N_j the number of markers found in plant j . The genetic dissimilarity was expressed by the formula $D_{ij} = 1 - S_{ij}$. The analysis was performed with a dissimilarity matrix using R PACKAGE 4.0 (Casgrain and Legendre, 2001).

With data on *Phyllonorycter* composition, a *Phyllonorycter* index (PI) was

TABLE 1. Leaf traits (mean ± SD) of pure populations of the two *Quercus* species.

Leaf traits	<i>Quercus crispula</i>		<i>Quercus dentata</i>
	Hamamasu	Toishiyama	Nakaotofuke
Dry mass (g)	0.35 ± 0.10	0.41 ± 0.14	1.22 ± 0.36
Area (cm ²)	80.7 ± 18.9	103.0 ± 29.5	192.4 ± 44.6
Perimeter (cm)	50.8 ± 5.88	60.6 ± 9.86	69.7 ± 10.0
Length (cm)	15.2 ± 1.63	17.1 ± 2.27	21.2 ± 2.50
Width (cm)	8.49 ± 1.15	9.63 ± 1.53	13.77 ± 1.63
Length/width proportion	1.82 ± 0.16	1.81 ± 0.15	1.57 ± 0.13
Number of lobes	22.6 ± 2.05	23.7 ± 2.29	16.7 ± 1.64
Leaf mass per area (mg/cm ²)	4.32 ± 0.55	3.97 ± 0.49	6.24 ± 0.53
Density of stellate hairs (hairs/mm ²)	0.32 ± 0.34	0.14 ± 0.21	3.63 ± 0.91
Length of stellate hairs (µm)	88.2 ± 70.4	72.8 ± 78.8	396.7 ± 41.8
Individuals with solitary hairs (%)	66	24	6
Individuals with short hairs (%)	88	70	100

calculated for each tree with the following formula, $PI = (P_D - P_C)/(P_D + P_C)$, where P_D is the number of individuals of two *Phyllonorycter* species specific to *Q. dentata* and P_C is the number of individuals of seven species specific to *Q. crispula*.

RESULTS

Morphological analyses—Morphological data for the three pure populations are given in Table 1. One-way ANOVA, Kruskal-Wallis, and χ^2 tests revealed significant differences in all leaf traits between the two species and in some traits among the three pure populations (Table 2). Factor analysis was per-

formed on these traits for the three pure populations (Table 3). Dry mass, area, perimeter, length, and width showed high loadings to Factor 1 in all populations. Therefore, only area was used among these five traits in the canonical discriminant analysis.

The discriminant analysis was made first for individuals from the three pure populations on the basis of area, length/width proportion, number of lobes, leaf mass per area (LMA), density of stellate hairs, length of stellate hairs, the presence or absence of solitary hairs, and the presence or absence of short hairs. In this analysis, the data for *Q. crispula* from the

TABLE 2. Results of one-way ANOVA, Kruskal-Wallis test, and χ^2 test for leaf traits of pure populations of *Quercus dentata* and *Q. crispula* (***) $P < 0.001$. T-K = Tukey-Kramer honestly significant difference (HSD) test.

Leaf traits	Source	df	Sum of squares or chi-square	Mean square	F statistics and/or P value	T-K ^a
Dry mass	Locality	2	23.676	11.838	221.095***	B
	Error	147	7.871	0.054		
	Total	149	31.546			
Area	Locality	2	349 960	174 980	163.360***	A
	Error	147	157 456	1071		
	Total	149	507 417			
Perimeter	Locality	2	8995	4497.64	58.241***	A
	Error	147	11 352	77.23		
	Total	149	20 347			
Length	Locality	2	957.8	478.896	102.063***	A
	Error	147	689.8	4.692		
	Total	149	1647.5			
Width	Locality	2	773.6	386.821	183.247***	A
	Error	147	310.3	2.111		
	Total	149	1083.9			
Length/width proportion	Locality	2	2.024	1.012	46.708***	B
	Error	147	3.185	0.022		
	Total	149	5.210			
Number of lobes	Locality	2	1394.2	697.113	172.838***	A
	Error	147	592.9	4.033		
	Total	149	1987.1			
Leaf mass per area	Locality	2	149.71	74.854	274.667***	A
	Error	147	40.06	0.273		
	Total	149	189.77			
Density of stellate hairs ^b		2	104.4		***	
Length of stellate hairs ^b		2	41.69		***	
Presence of solitary hairs ^c		2	46.15		***	
Presence of short hairs ^c		2	23.71		***	

^a A = significantly different between every pair of the populations; B = significantly different between *Q. dentata* (Nakaotofuke) and *Q. crispula* (Hamamasu and Toishiyama).

^b Kruskal-Wallis test.

^c χ^2 test.

TABLE 3. Results of factor analysis performed on pure populations of *Quercus crispula* and *Q. dentata*.

	<i>Q. crispula</i>		<i>Q. dentata</i>
	HM	TI	NO
Factor 1			
Eigenvalue	4.577	4.659	5.104
Contribution (%)	38.139	38.825	42.533
Factor loading			
Dry mass	0.871	0.842	0.986
Area	0.970	0.921	0.955
Perimeter	0.919	0.936	0.925
Length	0.928	0.937	0.868
Width	0.932	0.876	0.931
Length/width proportion	-0.353	-0.199	-0.093
Number of lobes	0.359	0.654	0.156
Leaf mass per area	0.178	0.203	0.701
Density of stellate hairs	-0.021	-0.135	0.096
Length of stellate hairs	0.048	-0.056	0.197
Presence of solitary hairs	0.090	-0.224	0.414
Presence of short hairs	-0.082	0.000	0.000
Factor 2			
Eigenvalue	1.588	1.532	1.593
Contribution (%)	13.235	12.770	13.274
Factor loading			
Dry mass	0.048	-0.059	-0.052
Area	0.035	-0.037	0.004
Perimeter	-0.034	-0.135	0.185
Length	-0.004	-0.084	0.381
Width	0.056	-0.057	-0.270
Length/width proportion	0.187	-0.084	0.301
Number of lobes	0.844	0.829	-0.433
Leaf mass per area	0.888	0.877	0.022
Density of stellate hairs	0.064	-0.100	-0.215
Length of stellate hairs	-0.118	-0.040	0.926
Presence of solitary hairs	0.155	-0.045	0.000
Presence of short hairs	0.049	0.149	0.395
Factor 3			
Eigenvalue	1.413	1.254	1.441
Contribution (%)	11.772	10.446	12.004
Factor loading			
Dry mass	0.020	-0.191	-0.014
Area	-0.065	-0.222	-0.044
Perimeter	0.046	-0.089	0.211
Length	0.242	0.042	0.138
Width	-0.346	-0.429	0.099
Length/width proportion	0.728	0.339	0.827
Number of lobes	0.058	-0.051	0.802
Leaf mass per area	-0.015	0.003	0.053
Density of stellate hairs	0.029	-0.003	0.170
Length of stellate hairs	0.832	0.920	-0.010
Presence of solitary hairs	-0.013	-0.083	0.000
Presence of short hairs	-0.017	0.055	-0.074

Note: HM = Hamamasu, TI = Toishiyama, NO = Nakaotofuke.

Toishiyama and Hamamasu populations were pooled, because the difference in each trait between these two populations was not large (Table 1). The first canonical axis explained 97.3% of the morphological variation. Table 4 gives canonical variate loadings. Area, number of lobes, LMA, density of stellate hairs, and length of stellate hairs showed high contributions to the canonical variate (CV). Figure 1 shows the distribution of CV scores. The two oaks *Q. crispula* and *Q. dentata* from the pure populations were clearly discriminated by CV scores.

Next, CV scores were calculated for individuals from the mixed stand at Ishikari using the discriminant formula obtained by the above analysis. Trees were divided into three

TABLE 4. Canonical variate (CV) loadings for each morphological trait.

Trait	CV
Area	0.836
Number of lobes	-0.849
Leaf mass per area	0.901
Length/width proportion	-0.638
Density of stellate hairs	0.965
Length of stellate hairs	0.939
Presence of solitary hairs	-0.404
Presence of short hairs	0.292

groups: *Q. crispula*-type (CV score < 0.1), *Q. dentata*-type (CV score > 0.4), and intermediate type (CV score 0.1–0.4) with reference to the distribution of CV scores in the pure populations (Fig. 1).

Genetic analysis—A total of 175 fragments ranging from approximately 100 to 400 base pairs (bp) were identified from individuals from Ishikari in the present analysis using the three primer pairs. Additional fragments were present but could not be scored either because of faint, inconsistent amplification or

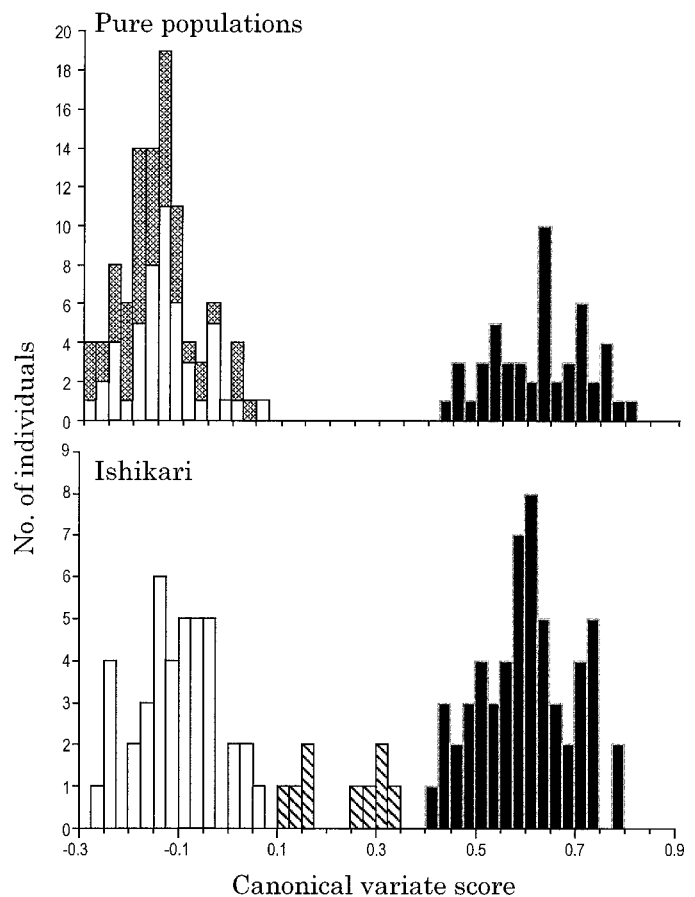


Fig. 1. Distribution of the first canonical variate (CV) scores based on morphological traits. Individuals from pure populations: black bars = *Quercus dentata* from Nakaotofuke, white bars = *Q. crispula* from Hamamasu, hatched bars = *Q. crispula* from Toishiyama. Individuals from Ishikari: black bars = putative *Q. dentata*, white bars = putative *Q. crispula*, hatched bars = morphologically intermediate individuals.

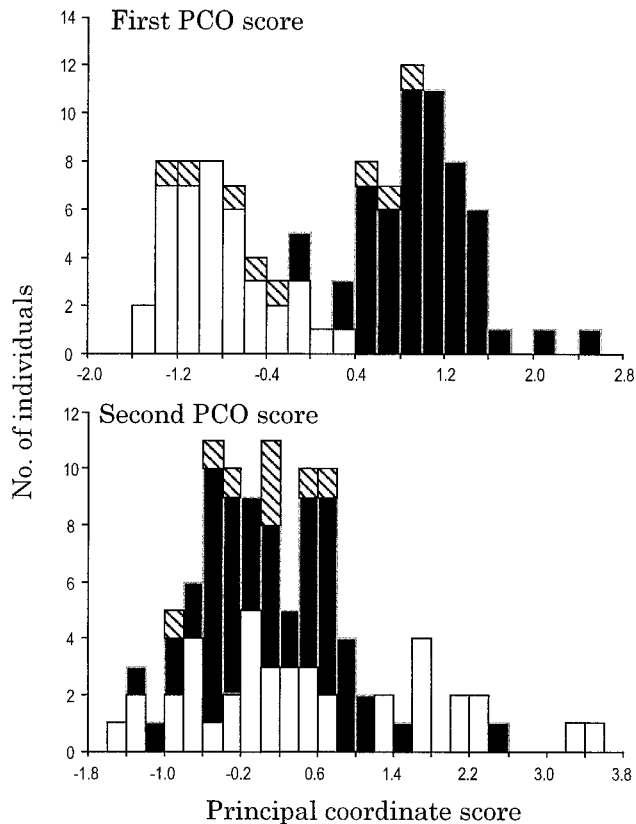


Fig. 2. Distribution of the first (A) and second (B) principal coordinate (PCO) scores based on AFLP data sets for individuals from Ishikari. Black bars = putative *Quercus dentata*, white bars = putative *Q. crispula*, hatched bars = morphologically intermediate individuals.

difficulty in differentiating two or more fragments of a similar mass.

The principal coordinate analysis was performed using these 175 markers for individuals from Ishikari. The first principal coordinate, which accounted for 6.7% of the variance, showed two clusters that discriminated between the two species, but individuals with intermediate morphological scores occurred in both clusters. The second principal coordinate, which accounted for 4.3% of the variance, did not separate the present oaks (Fig. 2). There was an association between the first PCO score in the AFLP analysis and the CV score in the morphological analysis (Fig. 3).

Phyllonorycter composition—A total of 2391 *Phyllonorycter* pupae were collected from 101 trees at Ishikari in 2000. Three individuals with -0.148 , -0.086 and -0.026 CV scores were dead or cut off by accidents, and no *Phyllonorycter* individual was collected on leaves from a tree with a CV score of 0.734. A total of 568 pupae were collected from 36 selected trees in 2001 (Table 5). The abundance of *Phyllonorycter* differed considerably between the two years; *P. pseudolautella* (on *Q. crispula*) and *P. leucocorona* (on *Q. dentata*) were abundant in 2000, but rare in 2001. Figure 4 shows the association between the *Phyllonorycter* index (PI) and the CV score in the morphological analysis. The PI was 1 in most individuals having high CV scores and -1 in those having low CV scores, although the association was somewhat weaker in 2001 than in 2000.

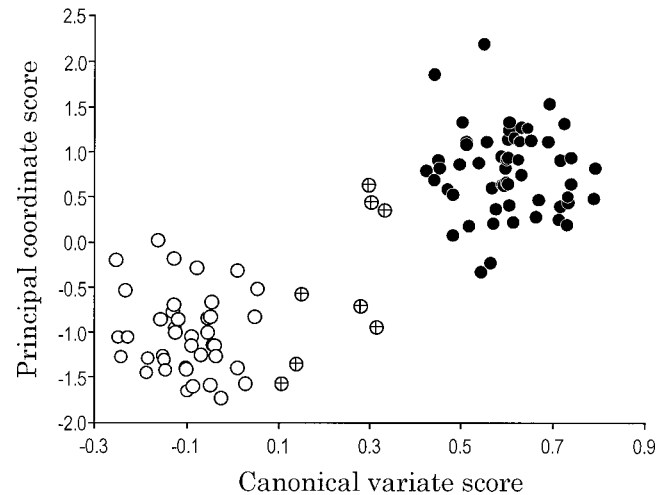


Fig. 3. Relation between the first canonical variate (CV) score in morphological analysis and the first principal coordinate (PCO) score in AFLP analysis. The overall relationship is highly significant ($P < 0.0001$). ●, putative *Quercus dentata*; ○, putative *Q. crispula*; ⊕, morphologically intermediate individuals.

Among the nine individuals with intermediate CV scores, one (individual G in Table 5) had a PI of 1 in both years. The PI values of the other eight individuals fell between -1 and 1 in either or both of the years (one oak tree having an intermediate PI value in 2000 were cut off by accident in spring in 2001, and then its PI value in 2001 could not be determined). Based on CV score and PI, the above eight individuals were tentatively assigned to be hybrids or have hybrid origins (e.g., backcross individuals), and one individual having PI score of 1 was presumed as *Q. dentata*. This individual also has a PCO score (0.433) typical of *Q. dentata* individuals. Thus, 105 individuals at Ishikari were tentatively classified into three categories: *Q. dentata* (57 individuals), *Q. crispula* (40 individuals), and hybrids or backcross individuals (eight individuals). Among these eight putative hybrids, five were found on the transect, and three were found among nine additional individuals.

DISCUSSION

Differentiating oak species is often very difficult because of continuous variation in morphological, ecological, and genetical traits due to shared ancient polymorphism and/or hybridization (Jensen et al., 1993; Kleinschmit et al., 1995; Bruschi et al., 2000; Tomlinson et al., 2000). This is true for *Quercus crispula* and *Q. dentata*, predominant members of cool-temperate forests of Japan. These two species differ in various morphological, ecological, and genetical traits (also see Ooba, 1989; Lee et al., 1996; Fujihara et al., 2001), and the density of stellate hairs and the characteristics of the acorn cap have been claimed as reliable criteria for their discrimination (Ooba, 1989). However, the detection of hybrids is not always possible only by these traits, because there is considerable within-species variation but only a small between-species gap. For example, the mean density of stellate hairs was 0–1.4 hairs/mm² in *Q. crispula* trees from pure stands at Toishiyama and Hamamasu and 2.2–6.8 hairs/mm² in *Q. dentata* trees from a pure stand at Nakaotofuke. If the density of stellate hairs shows intermediate inheritance, hybrids between an individual

TABLE 5. Number of *Phyllonorycter* moths (P_c = species specific to *Quercus crispula*, P_d = species specific to *Q. dentata*) on putative *Q. crispula*, putative *Q. dentata*, and morphologically intermediate individuals (A-I) in 2000 and 2001. Canonical variance (CV) and first principal coordinate (PCO1) scores are indicated.

Morphological type	No. of trees	P_c							P_d		CV	PCO1
		SI	AC	MO	PS	CR	PY	MA	LE	PE		
2000												
<i>Q. crispula</i>	37	601	23	4	248	8	3	128	1	2	-0.254-0.055	-1.737-0.051
Intermediate												
A	1	24	0	0	3	0	1	4	0	0	0.107	-1.563
B	1	7	1	0	0	0	0	2	0	1	0.137	-1.359
C	1	16	2	0	5	0	0	6	1	4	0.151	-0.599
D	1	35	3	0	1	5	0	5	0	1	0.169	—
E	1	16	1	0	4	0	1	7	0	0	0.275	-0.737
F	1	7	3	0	0	0	0	0	0	12	0.296	0.626
G	1	0	0	0	0	0	0	0	0	2	0.304	0.433
H	1	4	2	0	0	0	0	5	0	7	0.314	-0.890
I	1	1	1	0	0	0	0	2	0	5	0.332	0.345
<i>Q. dentata</i>	56	2	0	0	0	0	0	3	257	904	0.422-0.791	-0.231-2.308
2001												
<i>Q. crispula</i>	13	65	3	0	6	5	1	43	0	14		
Intermediate												
A	1	1	0	0	0	0	0	4	0	2		
B	1	3	0	0	0	0	0	1	0	2		
C	1	5	0	0	0	0	0	7	0	6		
D	1	6	0	0	0	0	0	2	0	1		
E	1	6	0	0	0	0	0	12	0	2		
G	1	0	0	0	0	0	0	0	0	12		
H	1	0	1	0	1	0	0	9	1	1		
I	1	4	0	0	0	1	0	0	0	14		
<i>Q. dentata</i>	15	0	0	0	0	0	0	6	7	314		

Note: SI = *P. similis*, AC = *P. acutissima*, MO = *P. mongolicae*, PS = *P. pseudolautella*, CR = *P. crenata*, PY = *P. pygmaea*, MA = *P. matsudai*, LE = *P. leucocorona*, PE = *P. persimilis*.

of *Q. crispula* with 1 hair/mm² and an individual of *Q. dentata* with 5 hairs/mm² are expected to have 3 hairs/mm², a score within the range of *Q. dentata*. In addition, hybrids may have characteristics of either of the parental species due to dominant or recessive inheritance. These problems can be solved by multivariate analysis that identifies linear combinations of variables. In this study, we carried out canonical discriminant analysis with eight morphological traits using pure populations of the two oak species as reference and revealed that nine out of 105 individuals in a mixed stand at Ishikari had intermediate morphology.

The composition of *Phyllonorycter* (leafminer) species was applied for the discrimination of hybrid oaks for the first time in this study. The *Phyllonorycter* composition analysis was consistent with the multivariate analysis of morphological traits for eight individuals among nine, suggesting the usefulness of the leafminer information for predicting hybridization. The utility of leafminers for the identification of hybrids relies on their high host specificity. In general, leafminers are known to have narrow host preferences (Schoonhoven et al., 1998). In fact, the present *Phyllonorycter* species seem to be specific to either of *Q. dentata* or *Q. crispula* (Sato, 1991; Fujihara et al., 2001). At Ishikari, however, some putative *Q. crispula* and *Q. dentata* were mined by the species that were not specific to the species. The *Phyllonorycter* species may not be complete in host selection, or leaf chemical characteristics of these oaks may have changed according to introgression (host selection of leafminers is expected to depend on chemical characteristics of leaves).

The PCOA using AFLP data revealed that *Q. dentata* and

Q. crispula have also differentiated at the molecular level. However, the degree of differentiation was low, and the AFLP data were less informative for the identification of hybrids. In addition, no diagnostic AFLP marker was obtained; i.e., markers that were observed in all individuals of one species were also observed in the other species at high frequencies (>0.95), and markers observed only in either of the two oak species were low in frequency (<0.16). Previous studies also reported that the degree of molecular differentiation is low between some sibling pairs; *Q. robur-Q. petraea* (Kleinschmit et al., 1995), *Q. grisea-Q. gambelii* (Howard et al., 1997) and *Q. petraea-Q. pubescent* (Bruschi et al., 2000).

Despite *Q. crispula* and *Q. dentata* hybridizing in nature, they remain morphologically, genetically, and ecologically distinct as do other sibling pairs of *Quercus* (Kleinschmit et al., 1995; Howard et al., 1997; Bruschi et al., 2000; Tomlinson et al., 2000). Jiggins and Mallet (2000) suggested that such bimodal hybrid zones are more effectively maintained by ecological divergence between parental species than by their genetic incompatibility. In fact, most pairs of *Quercus* species that remain distinct despite hybridization differ in ecological niches (Kleinschmit et al., 1995; Howard et al., 1997; Bruschi et al., 2000; Tomlinson et al., 2000; Williams et al., 2001). The difference in leaf traits (e.g., the density of stellate hairs or LMA) between the present study species would also reflect the difference in their adaptations to environmental conditions such as humidity or light regimes (Zhou et al., 1995; Lambers et al., 1998). In addition, Wu (2001) suggested that if reproductive isolation has once developed between species or populations to some degree, genes responsible for that isolation

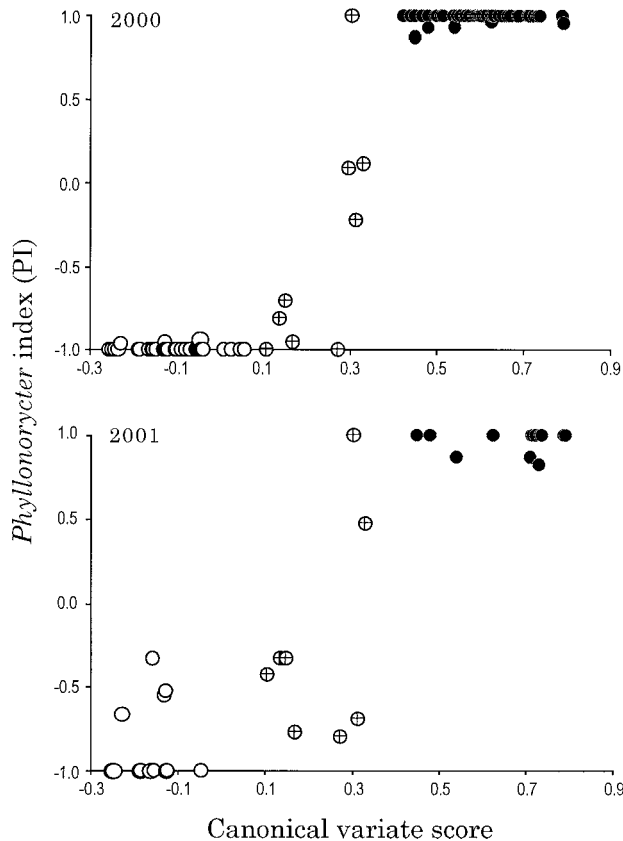


Fig. 4. Relation between the first canonical variate (CV) score in morphological analysis and *Phyllonorycter* index (PI) in 2000 and 2001. ●, putative *Quercus dentata*; ○, putative *Q. crispula*; ⊕, morphologically intermediate individuals.

and submitted to differential selection might not transfer across species even if hybridization occurs.

In conclusion, *Q. crispula* and *Q. dentata* were revealed to have differentiated in morphological traits, molecular (AFLP) markers, and composition of *Phyllonorycter* species. Morphological traits and *Phyllonorycter* composition were useful for the identification of hybrids, while AFLP data were less informative because the degree of molecular differentiation between the parental species was low. Out of 105 individuals from a mixed forest, nine were morphologically intermediate, and eight out of these nine individuals were also intermediate in the *Phyllonorycter* composition. These eight individuals were tentatively assigned to be hybrids or to have hybrid origins.

LITERATURE CITED

- AJMONEMARSAN, P., A. VALENTINI, M. CASSANDRO, G. VECCHIOTTIANTALDI, G. BERTONI, AND M. KUIPER. 1997. AFLP markers for DNA fingerprinting in cattle. *Animal Genetics* 28: 418–426.
- ANDERSON, E. 1949. *Introgressive hybridization*. John Wiley and Sons, New York, New York, USA.
- BODÉNÉS, C., S. JOANDET, F. LAIGRET, AND A. KREMER. 1997. Detection of genomic regions differentiating two closely related oak species *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. *Heredity* 78: 433–444.
- BRUSCHI, P., G. G. VENDRAMIN, F. BUSSOTTI, AND P. GROSSONI. 2000. Morphological and molecular differentiation between *Quercus petraea* (Matt.) Liebl. and *Quercus pubescens* Willd. (Fagaceae) in northern and central Italy. *Annals of Botany* 85: 325–333.

- BURGER, W. C. 1975. The species concept in *Quercus*. *Taxon* 24: 45–50.
- CASGRAIN, P., AND P. LEGENDRE. 2001. The R Package for multivariate and spatial analysis, version 4.0 d5, user's manual. Département de sciences biologiques, Université de Montréal, Montréal, Québec, Canada. Available at <http://www.fas.umontreal.ca/BIOL/legendre/>.
- CRESSWELL, A., N. R. SACKVILLE HAMILTON, A. K. ROY, AND B. M. F. VIEGAS. 2001. Use of amplified fragment length polymorphism markers to assess genetic diversity of *Lolium* species from Portugal. *Molecular Ecology* 10: 229–241.
- DICE, L. R. 1945. Measures of the amount of ecological association between species. *Ecology* 26: 297–302.
- DUMOLIN-LAPEGUE, S., B. DEMESURE, S. FINESCHI, V. LE CORRE, AND R. J. PETIT. 1997. Phylogeographic structure of white oaks throughout the European continent. *Genetics* 146: 1475–1487.
- FRITZ, R. S. 1999. Resistance of hybrid plants to herbivores: genes, environment, or both? *Ecology* 80: 382–391.
- FUJIHARA, J., H. SATO, AND T. KUMATA. 2001. The pupal cremasters as a diagnostic character for species of *Phyllonorycter* (Lepidoptera: Gracilariidae), with description of a new species of the *nipponicella* complex from Japan. *Insect Systematics and Evolution* 31: 387–400.
- HARDIN, J. W. 1975. Hybridization and introgression in *Quercus alba*. *Journal of Arnold Arboretum* 56: 336–363.
- HASHIZUME, H., Z. SUO, J. H. LEE, S. OKADA, AND F. YAMAMOTO. 1994. Fundamental studies on the breeding of *Quercus* species (II): on the characters of leaves and fruits in natural hybrids among *Q. dentata*, *Q. serrata* and *Q. mongolica* var. *grosseserrata*. *Monograph Collection of Japanese Forest Society* 105: 325–328 (in Japanese).
- HILL, M., H. WITSEBOER, M. ZABEAU, P. VOS, R. KESSELI, AND R. MICHELMORE. 1996. PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. *Theoretical and Applied Genetics* 93: 1202–1210.
- HOWARD, D. J., R. W. PRESZLER, J. WILLIAMS, S. FENCHEL, AND W. J. BOECKLEN. 1997. How discrete are oak species? Insights from a hybrid zone between *Quercus grisea* and *Quercus gambelii*. *Evolution* 51: 747–755.
- JENSEN, R. J., S. C. HOKANSON, J. G. ISEBRANDS, AND J. F. HANCOCK. 1993. Morphometric variation in oaks of the Apostle Islands in Wisconsin: evidence of hybridization between *Quercus rubra* and *Q. ellipsoidalis* (Fagaceae). *American Journal of Botany* 80: 1358–1366.
- JIGGINS, C. D., AND J. MALLET. 2000. Bimodal hybrid zones and speciation. *Trends in Ecology and Evolution* 15: 250–255.
- KIM, M. H., H. S. SONG, AND C. S. KIM. 1992. Morphological types and seasonal loss of the trichome of some *Quercus* species in Korea. *Korean Journal of Plant Taxonomy* 22: 13–21.
- KLEINSCHMIT, J. R. G., R. BACILIERI, A. KREMER, AND A. ROLOFF. 1995. Comparison of morphological and genetic traits of pedunculate oak (*Q. robur* L.) and sessile oak (*Q. petraea* (Matt.) Liebl.). *Silvae Genetica* 44: 256–269.
- LAMBERS, H., F. S. CHAPIN, III, AND T. L. PONS. 1998. *Plant physiological ecology*. Springer-Verlag, New York, New York, USA.
- LEE, J. H., H. HASHIZUME, AND F. YAMAMOTO. 1996. Variations in the flowering time, pollen morphology and fertility of *Quercus dentata* Thunb., *Q. serrata* Thunb., *Q. mongolica* Fischer var. *grosseserrata* Rehder et Wilson and their intermediate types. *Journal of Japanese Forest Society* 78: 452–456 (in Japanese with English summary).
- LU, J., M. R. KNOX, M. J. AMBROSE, J. K. M. BROWN, AND T. H. N. ELLIS. 1996. Comparative analysis of genetic diversity in pea assessed by RFLP- and PCR-based methods. *Theoretical and Applied Genetics* 93: 1103–1111.
- MIZAZAKI, Y. 1989. Ecological genetic studies of *Quercus crispula* in Hokkaido. *Forest and Tree Breeding* 153: 1–5 (in Japanese with English summary).
- O'HANLON, P. C., R. PEAKALL, AND D. T. BRIESE. 1999. Amplified fragment length polymorphism (AFLP) reveals introgression in weedy *Onopordum* thistles: hybridization and invasion. *Molecular Ecology* 8: 1239–1246.
- Ooba, H. 1989. Fagaceae. In Y. Satake, H. Hara, S. Watari, and T. Tominari [eds.], *Wild flowers of Japan: woody plants 1*, 66–75. Heibonsha, Tokyo, Japan (in Japanese).
- ORIANI, C. M. 2000. The effects of hybridization in plants on secondary chemistry: implications for the ecology and evolution of plant–herbivore interactions. *American Journal of Botany* 87: 1749–1756.
- RIESEBERG, L. H., AND J. F. WENDEL. 1993. Introgression and its conse-

- quences in plants. In R. G. Harrison [ed.], Hybrid zones and the evolutionary process, 70–109. Oxford University Press, Oxford, UK.
- SAMUEL, R., W. PINSKER, AND F. EHRENDORFER. 1995. Electrophoretic analysis of genetic variation within and between populations of *Quercus ceris*, *Q. pubescens*, *Q. petraea* and *Q. robur* (Fagaceae) from Eastern Austria. *Botanical Acta* 108: 290–299.
- SATO, H. 1991. Differential resource utilization and co-occurrence of leaf miners on oak (*Quercus dentata*). *Ecological Entomology* 16: 105–113.
- SCHOONHOVEN, L. M., T. JERMY, AND J. J. A. VAN LOON. 1998. Insect-plant biology: from physiology to evolution. Chapman and Hall, London, UK.
- SHARMA, S. K., M. R. KNOX, AND T. H. N. ELLIS. 1996. AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPD analysis. *Theoretical and Applied Genetics* 93: 751–758.
- SHIBATA, S., T. A. ISHIDA, F. SOEYA, N. MORINO, K. YOSHIDA, H. SATO, AND M. T. KIMURA. 2001. Within-tree variation in density and survival of leafminers on oak *Quercus dentata*. *Ecological Research* 16: 135–143.
- TOMLINSON, P. T., R. J. JENSEN, AND J. F. HANCOCK. 2000. Do whole tree silvic characters indicate hybridization in red oak (*Quercus* Section *Lobatae*)? *American Midland Naturalist* 143: 154–168.
- VAN VALEN, L. 1976. Ecological species, multispecies, and oaks. *Taxon* 2/3: 233–239.
- WILLIAMS, J. H., W. J. BOECKLEN, AND D. J. HOWARD. 2001. Reproductive processes in two oak (*Quercus*) contact zones with different levels of hybridization. *Heredity* 87: 680–690.
- WU, C.-I. 2001. The genetic view of the process of speciation. *Journal of Evolutionary Biology* 14: 851–865.
- YOUNG, W. P., C. O. OSTBERG, P. KEIM, AND G. H. THORGAARD. 2001. Genetic characterization of hybridization and introgression between anadromous rainbow trout (*Oncorhynchus mykiss irideus*) and coastal cutthroat trout (*O. clarki clarki*). *Molecular Ecology* 10: 921–930.
- ZHOU, Z. K., H. WILKINSON, AND Z. Y. WU. 1995. Taxonomical and evolutionary implications of the leaf anatomy and architecture of *Quercus* L. subgenus *Quercus* from China. *Cathaya* 7: 1–34.