Phylogenetic relationship among the section *Stenophora* in the genus *Dioscorea* based on the analysis of nucleotide sequence variation in the phosphoglucose isomerase (*Pgi*) locus

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To determine the phylogenetic relationship of six species (*Dioscorea gracillima*, *D. nipponica*, *D. quinqueloba*, *D. septemloba*, *D. tenuipes*, and *D. tokoro*) in the section of *Stenophora* in the genus of *Dioscorea*, nucleotide sequence variation in 1073 bp of coding region of the phosphoglucose isomerase (*Pgi*) locus was analyzed. Estimates of nucleotide diversity (π) for *D. gracillima*, *D. quinqueloba*, *D. tenuipes*, and *D. tokoro* were 0.0020, 0.0005, 0.0025, and 0.0013, respectively. Deviation from neutrality in the pattern of polymorphism in these four species was not detected by the Tajima's test. The difference in the pattern of amino acid substitutions between divergence and polymorphism was not observed by McDonald and Kreitman test. The obtained phylogenetic trees indicated that *D. tokoro* and *D. tenuipes* belonged to a monophyletic clade, while the other four species formed a separate monophyletic group. The branch lengths of phylogenetic tree varied among species. *D. nipponica* had a significantly faster substitution rate compared to *D. gracillima*, *D. quinqueloba*, and *D. septemloba*.

INTRODUCTION

The genus *Dioscorea*, which contains about 600 species (Coursey, 1967), is the largest in the family of yam, Dioscoreaceae (Burkill, 1960). Some *Dioscorea* species are economically important in many regions over the world, especially in southeastern Asia and Africa (Alexander and Coursey, 1969). In Japan, *D. opposita* (nagaimo) is cultivated widely. Phylogenetic relationship of this genus has not been well examined because of difficulty in species identification due to a high level of polymorphism with respect to morphological characters (Okagami, 1985). Some species have two different species names for male and female plants, because all species in this genus are dioecious (Okagami, 1985).

The genus *Dioscorea* in the Old World was classified into 23 sections (Burkill, 1960). One of the sections, *Stenophora* has many ancestral characters such as rhizome. Fossil record of *Stenophora* is the oldest in the genus *Dioscorea*.

Therefore, this section is considered as one of the oldest in the genus *Dioscorea* (Burkill, 1960). Eight species of the *Stenophora* are naturally occurring in Japan (except Ryukyu). Among the eight species, *D. septemloba*, *D. nipponica*, and *D. gracillima* are distributed only in northern part of Japan, and *D. tokoro*, *D. tenuipes*, and *D. quinqueloba* are distributed almost all over Japan. The other two species have restricted distribution area. *D. izuensis* inhabits only in the Izu peninsula, and *D. asclepiadea* inhabits only in the southern part of Kyushu island.

In this study, nucleotide sequence variation of the phosphoglucose isomerase (*Pgi*) locus was analyzed. PGI (EC. 5.3.1.9) catalyzes the reversible isomerization of 6-phosphoglucose and 6-phosphofructose. This is an essential metabolic reaction that precedes the sucrose biosynthesis in the cytosol. Previous allozyme studies on PGI of various organisms (reviewed in Riddoch, 1993) suggested that the PGI allozyme variation could be adaptive. The allozyme variation of PGI (for example: butterfly *Colias*: Watt, 1977; sea anemone *Metridium*: Hoffmann, 1981; blue mussel *Mytilus*: Hall, 1985; amphipod *Gammarus*: Patarnello et al., 1992) showed latitudinal clines, suggesting the association between PGI allozyme and tempera-

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ture. In addition, overdominance under certain temperature conditions (butterfly: Watt, 1977; amphipod: Patarnello et al., 1992) and directional selection for high temperature (sea anemone: Hoffmann, 1981; blue mussel: Hall, 1985) were detected.

A Japanese species *D. tokoro* in the section *Stenophora* has been investigated for the *Pgi* locus with respect to allozyme (Terauchi, 1990) and DNA (Terauchi et al., 1997) variations. The allozyme study of *D. tokoro* (Terauchi, 1990) showed that three major alleles (a, b, and c) of PGI existed at high frequencies in natural populations in Japan. Since allele frequencies of PGI allozymes of *D. tokoro* also show latitudinal cline, a possibility of natural selection related to temperature difference was suggested (Terauchi, 1990). However, it was shown that these three alleles were related to each other with only one replacement nucleotide substitutions, that sequence variation of the *Pgi* region in *D. tokoro* was low, and that there was an excess of replacement nucleotide polymorphism (Terauchi et al., 1997). These observed patterns of DNA and allozyme variation were explained by a neutral mutation hypothesis with the assumption of small effective size of *D. tokoro* population, without invoking balancing selection (Terauchi et al., 1997).

One of the purposes of this study is to investigate phylogenetic relationship between species of the section *Stenophora* based on nucleotide sequence variation of the *Pgi* locus. For the present study, six species, *D. gracillima*, *D. nipponica*, *D. quinqueloba*, *D. septemloba*, *D. tenuipes*, and *D. tokoro* were investigated. Because of difficulties in sampling, *D. izuensis* and *D. asclepiadea* were not included. The second purpose is to investigate the level and pattern of nucleotide variation at the *Pgi* locus within and between species of the section *Stenophora*. As mentioned, investigated six *Dioscorea* species have different distribution patterns, i. e., area and locality. If the PGI polymorphism is related to the adaptation of these *Dioscorea* species to temperature condition, the difference in the distribution of investigated species may be reflected in the pattern of nucleotide variation at the *Pgi* locus.

MATERIALS AND METHODS

Plant materials. Six species (*D. gracillima* Miq., *D. nipponica* Makino, *D. quinqueloba* Thunb., *D. septemloba* Thunb., *D. tenuipes* Franch. et Savat., and *D. tokoro* Makino) were investigated in this study. To estimate the level of polymorphism, two plants each were used for three of the species (*D. tenuipes*, *D. quinqueloba*, and *D. gracillima*). Since each individual was heterozygous at the DNA level at the *Pgi* locus, the number of sequences considered was four to obtain estimate of polymorphism for these three species. One plant each was used for the other species. All the nine plants were grown in a nursery from rhizomes which were collected from the locations shown in Table 1.

Reverse Transcription-PCR and direct sequencing.

In the present study, cDNA sequence was determined because the genomic *Pgi* gene is too long to investigate the entire region (about six kb in *Clarkia*). By using cDNA sequence, the expressed *Pgi* genes were analyzed. The procedure of cDNA synthesis followed that described for *D. tokoro* (Terauchi et al., 1997). Fresh leaves (0.5 g) were obtained from each plant, from which total RNA was extracted, using AGPC (acid guanidinium thiocyanate-phenol-chloroform) method (Chomczynski and Sacchi, 1987). cDNA was synthesized from 2 µg of total RNA by oligo-dT primed reverse transcription, using Ready-To-Go T-Primed First-Strand Kit (Pharmacia). The *Pgi* gene was amplified from cDNA by 30 cycles of PCR, each of cycles consisting of 1 min at 94°C (denaturing), 2 min at 55°C (annealing) and 3 min at 72°C (extension). Because of nucleotide substitutions among investigated species, primers designed for the 5' and 3' ends of *D. tokoro* cDNA sequence failed the PCR amplification of the entire *Pgi* of these species. Instead, we used two primers, 95.1 and 93.12 (Fig. 1) to amplify a part of the *Pgi* for these species. These primers were originally designed for *D. tokoro* *Pgi* cDNA sequence at nucleotide position 199–217 and 1326–1307, respectively (Terauchi et al., 1997). The length of the PCR product obtained from this set of primers was expected to be 1073

Table 1. List of plant materials used for this study

Species	Accession number	Geographic origin	
		Place	Prefecture
<i>D. tokoro</i>	DT37	unknown	Wakayama
<i>D. tenuipes</i>	DTE1	Takinohai	Wakayama
	DTE2	Nishino	Shiga
<i>D. septemloba</i>	DSE1	Mt. Ooe	Kyoto
<i>D. nipponica</i>	DN1	Iide	Niigata
<i>D. quinqueloba</i>	DQ1	Chikatsuyu	Wakayama
	DQ2	Amagase, Uji	Kyoto
<i>D. gracillima</i>	DGR1	Koyaoka, Ayabe	Kyoto
	DGR2	Shuzan, Keihoku	Kyoto

Fig. 1. Positions and nucleotide sequences of primers used for the *Pgi* region in this study. An arrow corresponds to the DNA region sequenced by a primer. Nucleotide position of the 5' end of primer is shown beside each arrow. Coordinates of the *Pgi* coding sequence follows Terauchi et al. (1997). Length of each primer is shown in parenthesis. Sequenced region is shown as hatched box. The exon structure is indicated.

bp long excluding the primer length. First, PCR amplification was performed in a 50 μ l reaction, containing total cDNA as template, 0.1 mM each of dNTPs, 1 unit of Taq DNA polymerase (Sawady), and 50 pM of each primer. Using 2 μ l of the first PCR product as template, PCR was repeated in a 100 μ l reaction volume containing a double amount of reagents. The second PCR product was purified by glass powder using the GeneClean II Kit (Bio101 Inc.). Purified DNAs were directly sequenced by the dideoxy chain termination method (Sanger et al., 1977) using PRISM: Dye Terminator Cycle Sequencing KIT (ABI). To sequence both strands of the 1073-bp region, nine primers (Fig. 1) were used. Some nucleotide positions were represented by two nucleotide signals, which may be heterozygous nucleotide sites, because all the species in the genus *Dioscorea* are outcrossing. Sites were assumed as heterozygous only when two nucleotide signals were observed in both reverse and forward sequencing reactions. The *Pgi* sequences of *D. tokoro* (DT37), *D. tenuipes* (DTE1), and *D. quinqueloba* (DQ1) were obtained from Terauchi et al. (1997). Newly obtained *Pgi* sequences were deposited in DDBJ data base under the accession number AB006615-AB006620.

Sequence analysis. To quantify the level of nucleotide polymorphism, nucleotide diversity (π : Nei and Li, 1979; Tajima and Nei, 1984) of *D. gracillima*, *D. septemloba*, and *D. tenuipes* was estimated. Tajima's test (1989) were conducted to examine the neutrality of DNA polymorphism. To conduct the intra- and interspecific comparisons of amino acid substitutions, McDonald and Kreitman's test (1991) was performed. To quantify the level of divergence between species, genetic distance was calculated by Jukes and Cantor method (d: 1969) using PHYLIP ver. 3.572 (Felsenstein, 1995) and Nei and Gojobori method (d_N and

d_S : 1986) using ODN (Ina, 1992). In the above sequence analysis, we assume that heterozygous sites were due to allelic variation. Among the six species investigated, *D. tokoro* is diploid, and was shown to have a single *Pgi* locus per genome (Terauchi et al., 1997). On the other hand, we do not have any clear information on the polyploidy and copy number of the *Pgi* genes for the other five species. Therefore, estimated values of polymorphism and divergence for these five species may be biased from true values, since allelic comparison may not be done in this analysis.

Phylogenetic analysis. Nucleotide and deduced amino acid sequences of nine plants were used in phylogenetic analysis. Phylogenetic trees were constructed by neighbor-joining (NJ) method (Saitou and Nei, 1987) and a parsimony method (Fitch, 1971). NJ tree was constructed by using the Neighbor program of PHYLIP ver. 3.572 (Felsenstein, 1995) based on the genetic distances (d) between species calculated by Jukes and Cantor method (1969). Parsimony tree was constructed by using the PAUP (Swofford, 1993) with heuristic search option. The bootstrap probabilities for both NJ and parsimony trees were estimated for each internal branch by 100 replications. By constructing a NJ tree based on amino acid sequences of the six species and rice (as an outgroup), the root was determined for trees of nucleotide sequences. The reason for using amino acid sequence data was that synonymous nucleotide substitutions seem to be saturated between *Dioscorea* species and rice. Genetic distances for amino acid sequences were estimated by using Dayhoff PAM matrix (Dayhoff et al., 1979) in the PROTDIST program of PHYLIP ver. 3.572 (Felsenstein, 1995). To test the constancy of molecular evolutionary rate among lineages, Tajima's relative rate test (1993) was performed. This method is based on the chi-square test by comparing the

Table 2. Summary of variable sites in the investigated *Pgi* region of the section *Stenophola*

Site	222	234	266	270	285	303	318	320	325	336	354	357	365	378	393	395	408	426
Consensus	A	C	G/C	G	G	A	G	T/V	G/E	A	C	T	T/V	C	G/E	G/R	C	T
DT37	*	*	*	A	*	*	*	C/S	A/K	*	T	G	*	*	*	*	*	*
DTE1	C	*	*	A	*	*	*	*	A/K	*	T	G	C/A	*	*	*	*	*
DTE2	*	*	*	A	*	*	*	*	A/K	*	T	G	TC/A	*	*	*	*	*
DSE1	*	*	*	*	*	AG	*	*	*	*	*	*	*	*	*	*	AC	*
DN1	*	T	C/S	*	*	*	A	*	*	G	*	*	*	*	T/D	*	*	*
DQ1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C
DQ2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C
DGR1	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	T/I	*	*
DGR2	*	*	*	*	T	*	*	*	*	*	*	*	*	T	*	T/I	*	*

Table 2. Continued (1)

432	448	453	456	458	462	498	510	531	549	552	594	595	639	642	648	660	698	702	721
G	G/V	A	A	G/G	T	C	T	C	G	A	C	G/A	G	C	A	T	C/A	T	G/A
*	*	AG	*	*	C	T	A	*	*	*	*	A/T	*	T	*	*	*	T/S	*
*	*	*	*	*	C	T	*	*	*	*	*	*	*	T	*	*	*	C	*
*	*	*	*	*	*	*	*	T	*	*	*	*	A	*	*	*	G/G	*	*
*	A/M	*	T	*	*	*	*	T	A	*	T	*	*	*	T	A	G/G	*	*
A	*	*	G	*	*	*	*	*	G	*	*	*	*	*	*	*	G/G	*	*
A	*	*	G	A/D	*	*	*	*	G	*	*	*	*	*	*	*	G/G	*	*
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G/G	*	*
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G/G	*	*

Table 2. Continued (2)

745	751	774	789	807	819	825	840	855	856	861	891	900	903	906	909	917	926	927	932
T	C	T	T	C	C	T	A	T	T	T	A	T/N	A	T	A	C	C/T	T	T
C	T	*	*	*	*	*	*	*	C	G	G	*	*	*	*	*	*	*	*
*	T	A	*	*	*	*	G	*	*	*	*	*	G	A	*	*	*	*	*
*	*	A	*	*	*	*	G	*	*	*	*	*	*	A	*	*	*	*	*
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	*	*	T	T	*	*	*	*	*	*	*	*	*	T	T	T/I	*	*
*	*	*	C	*	T	*	*	*	*	*	*	G/K	*	*	*	T	*	*	*
*	*	*	C	*	T	*	*	*	*	*	*	G/K	*	*	*	T	*	*	*
*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	A	C
*	*	*	*	*	*	C	*	GT	*	*	*	*	*	*	*	*	*	*	C

Table 2. Continued (3)

963	964	1006	1041	1052	1062	1064	1098	1099	1111	1119	1129	1153	1160	1185	1228	1263	1269	1272	1287
A	T	C/R	C	C/A	T	A	C	T/S	A/I	T	A/T	C/P	A/T	A	G/V	A	G	A	G
*	*	*	*	*	*	*	*	*	G/V	*	*	*	*	*	*	*	T	*	*
*	*	*	*	*	*	*	*	A/T	G/V	*	*	T/S	*	*	A/I	*	*	*	*
*	*	*	*	*	*	*	*	*	G/V	*	*	*	*	*	A/I	*	*	*	*
*	*	G/G	*	*	*	AC	*	*	T/F	G	*	*	*	G	*	G	*	*	*
*	C	G/G	*	G/G	A	*	G	*	*	*	G/A	*	C/P	*	*	*	*	AG	*
*	*	G/G	T	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	G/G	T	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AG	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A
G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A

Asterisk means the same nucleotide as the consensus. Two letters are used in the site that is heterozygous. In the case of replacement substitutions, one letter code of amino acid is shown after the slash.

total number of different sites in aligned three sequences. In this test, there are two different methods, 1D and 2D methods. The 1D method was to compare the number of different sites among compared two species, while the 2D method was to compare the number of different sites by separating transitions and transversions among compared two species. The 2D method provides a more reliable test result to detect the difference in evolutionary rate if rates of transition and transversion are different (Tajima, 1993).

RESULTS

Sequence variation in the *Pgi* locus among species in the section *Stenophora*. The summary of variable sites is shown in Table 2. There are 78 variable sites in the sample of nine individuals of six species (nine sequences). All these differences were caused by nucleotide substitutions. No length variations and frame shifts were

detected. Of these sites, 15 sites were detected within species (eleven are synonymous and four are replacement substitutions) and 63 sites were found between species (46 are synonymous and 17 are replacement substitutions). The *Pgi* sequences of rice (Nozue et al., 1996), *Arabidopsis thaliana* (Thomas et al., 1993), and *Clarkia lewisii* (Thomas et al., 1992) can be aligned with the *Dioscorea* sequences. There is no change in the size of the reading frame in the investigated region among these distant species.

The levels of polymorphism within species and divergence between species are summarized in Tables 3 and 4. Although *D. quinqueloba* has a relatively low nucleotide diversity, estimates of the other three species are similar to each other. But these estimates are lower than those of the coding regions in nuclear genes in other plant species (*C. lewisii* (*Pgi*), 0.0053; Thomas et al., 1993; *Arabis gemmifera* (*Adh*), 0.0055; Miyashita et al., 1996; *A. thaliana* (*Adh*),

Table 3. Summary of polymorphism in the *Pgi* locus of *Dioscorea* species

Species		π	Tajima's D	The number of polymorphic sites	
				syn	rep
<i>D. tokoro</i> *	(n = 20)	0.0015	0.3225	2	4
<i>D. tenuipes</i>	(n = 4)	0.0030	1.5406	2	3
<i>D. quinqueloba</i>	(n = 4)	0.0006	1.6330	0	1
<i>D. gracillima</i>	(n = 4)	0.0022	0.6501	4	0
<i>D. septemloba</i>		(0.0028) [†]		3	0
<i>D. nipponica</i>		(0.0009) [†]		1	0

π is the nucleotide diversity (Nei and Li, 1979; Tajima and Nei, 1984), Tajima's D is the statistics of Tajima's test (1989), and n is the number of alleles compared.

* Recalculated from Terauchi et al. (1997), [†] The proportion of heterozygous sites.

Table 4. Summary of divergence in the *Pgi* locus between species

Comparison of species		Genetic distances*				The number of fixed substitutions	
		d	d _N	d _S	d _N /d _S	syn	rep
<i>D. tokoro</i>	vs <i>D. tenuipes</i>	0.0184	0.0073	0.0575	0.127	11	4
<i>D. tokoro</i>	vs <i>D. septemloba</i>	0.0218	0.0073	0.0704	0.104	16	6
<i>D. tokoro</i>	vs <i>D. nipponica</i>	0.0402	0.0174	0.1198	0.145	26	14
<i>D. tokoro</i>	vs <i>D. quinqueloba</i>	0.0258	0.0104	0.0881	0.118	21	8
<i>D. tokoro</i>	vs <i>D. gracillima</i>	0.0252	0.0086	0.0835	0.103	16	7
<i>D. tenuipes</i>	vs <i>D. septemloba</i>	0.0232	0.0098	0.0706	0.139	16	6
<i>D. tenuipes</i>	vs <i>D. nipponica</i>	0.0398	0.0172	0.1201	0.143	27	12
<i>D. tenuipes</i>	vs <i>D. quinqueloba</i>	0.0280	0.0104	0.0883	0.118	20	6
<i>D. tenuipes</i>	vs <i>D. gracillima</i>	0.0227	0.0089	0.0738	0.121	15	5
<i>D. septemloba</i>	vs <i>D. nipponica</i>	0.0266	0.0110	0.0794	0.139	19	9
<i>D. septemloba</i>	vs <i>D. quinqueloba</i>	0.0170	0.0043	0.0577	0.075	14	3
<i>D. septemloba</i>	vs <i>D. gracillima</i>	0.0137	0.0049	0.0449	0.109	9	4
<i>D. nipponica</i>	vs <i>D. quinqueloba</i>	0.0266	0.0104	0.0795	0.131	19	8
<i>D. nipponica</i>	vs <i>D. gracillima</i>	0.0290	0.0110	0.0927	0.119	19	9
<i>D. quinqueloba</i>	vs <i>D. gracillima</i>	0.0175	0.0043	0.0620	0.069	12	4

* d, the average number of substitutions per site, by Jukes and Cantor method (1969); d_N, the number of nonsynonymous substitutions per site; and d_S, the number of synonymous substitutions per sites. d_N and d_S were calculated by the method of Nei and Gojobori (1986).

Fig. 2. Phylogenetic trees based on the nucleotide and amino acid sequence data of the *Pgi* region. The bootstrap probability for internal branch is shown in parenthesis. Accession number is shown in parenthesis after species name. (A) The most parsimonious tree. The number of changes is shown on branch. Consistency index and retention index are 0.950, and 0.917, respectively. (B) NJ tree based on nucleotide sequences of the *Pgi*. Branch length is shown above branch. (C) NJ tree based on deduced amino acid sequence data. Branch length is shown above branch.

0.0056; Innan et al., 1996). Tajima's D value for *D. tokoro*, *D. tenuipes*, *D. quinqueloba* and *D. gracillima* in 1073-bp region were 0.3225, 1.5406, 1.6330, and 0.6501, respectively. None of the tests were significant. This result indicates that the pattern of nucleotide polymorphism does not deviate from the test assumptions, namely neutrality and population equilibrium, in any of these species. It should be mentioned, however, that these tests may not have enough power to detect the deviation from the test assumptions, because of small sample size.

D. gracillima, *D. septemloba*, and *D. nipponica* have only synonymous polymorphic sites, while *D. tokoro*, *D. tenuipes*, and *D. quinqueloba* have more replacement polymorphic sites than synonymous polymorphic sites. All the interspecific comparisons have two to five folds larger synonymous substitutions than replacement substitutions. To examine differences in the pattern of amino acid substitutions within and between species, MK test (McDonald and Kreitman, 1991) was performed. As for the divergence, interspecific comparison was done between *Dioscorea* species, and polymorphism data were obtained for the four *Dioscorea* species. All possible combinations of between and within species comparisons were examined by MK test, but none of comparisons were significant. The results indicated no significant difference in the pattern of amino acid substitutions in the *Pgi* coding region within and between *Dioscorea* species.

All the d_N/d_S ratios are much smaller than one (Table 4). There is no indication of adaptive evolution at the *Pgi* locus of *Stenophora* species investigated. It is noted that genetic distances between *D. nipponica* and any other *Dioscorea* species are relatively larger than the other (Table 4). However, the d_N/d_S ratios for comparison involving with

D. nipponica do not seem to be markedly larger than others, because both d_N and d_S for *D. nipponica* are large.

Phylogenetic analysis. Figure 2 shows the phylogenetic trees of the investigated *Dioscorea* species based on the *Pgi* sequence variations. Figure 2A is the tree obtained by a parsimony analysis by using PAUP. The parsimony analysis of nucleotide sequences of the 1073-bp *Pgi* region in *Dioscorea* yielded only one most parsimonious tree with 80 steps. Figure 2B is the tree obtained by NJ method. These two trees obtained by using two different methods resulted in identical topology with two clades. One clade contains *D. tokoro* and *D. tenuipes* (clade I), and the other clade contains the other four species (clade II). Since this clustering was supported by 100% bootstrap probability for both trees, this topology could be reliable.

The NJ tree of amino acid sequence data (Fig. 2C) indicated that the root of the *Stenophora* species was located at the branch between the two clades. Of these two clades, one clade which consists of *D. tokoro* and *D. tenuipes* (clade I) was supported by a high bootstrap probability (91%), but the other clade consisting of the other four species was not well supported (48% bootstrap probability). This result indicated that *D. tokoro* and *D. tenuipes* are monophyletic.

Test of constancy of evolutionary rate in the *Pgi* of *Dioscorea*. The phylogenetic trees (Fig. 2) show that the branch leading to *D. nipponica* is larger than the other branches. To test difference in evolutionary rate among species especially for *D. nipponica*, Tajima's relative rate test (Tajima, 1993) was conducted (Table 5). The outgroup species which are necessary for this test were determined from the topology of the NJ tree of amino acid sequences

Table 5. Results of relative rate test for the *Pgi* locus in *Dioscorea* species investigated in this study

Species compared		Outgroup	No. of different sites ^a									Chi-square	
1	2	3	m1	(s1	, v1)	m2	(s2	, v2)	m3	(s3	, v3)	1D	2D
<i>D. tokoro</i>	<i>D. tenuipes</i>	<i>D. septemloba</i>	8.5	4.5	4	9.8	6.8	3	16.5	10.5	6	0.09	0.59
<i>D. tokoro</i>	<i>D. tenuipes</i>	<i>D. nipponica</i>	9.5	5.5	4	8.8	5.8	3	33.5	20.5	13	0.03	0.15
<i>D. tokoro</i>	<i>D. tenuipes</i>	<i>D. quinqueloba</i>	9.5	5.5	4	8.8	5.8	3	20.5	16.5	4	0.03	0.15
<i>D. tokoro</i>	<i>D. tenuipes</i>	<i>D. gracillima</i>	9.5	5.5	4	8.8	5.8	3	16.0	11.2	4.8	0.03	0.15
<i>D. septemloba</i>	<i>D. nipponica</i>	<i>D. tokoro</i>	8.5	5.5	3	22.5	12.5	10	18.5	11.5	7	6.32 ^b	6.49 ^b
<i>D. septemloba</i>	<i>D. quinqueloba</i>	<i>D. tokoro</i>	8.5	5.5	3	10.5	9.5	1	18.5	11.5	7	0.21	2.07
<i>D. septemloba</i>	<i>D. gracillima</i>	<i>D. tokoro</i>	7.5	3.5	4	9	6.2	2.8	17.5	11.5	6	0.14	1.01
<i>D. nipponica</i>	<i>D. quinqueloba</i>	<i>D. tokoro</i>	21.5	11.5	10	8.5	7.5	1	21.5	14.5	7	5.63 ^b	8.21 ^b
<i>D. nipponica</i>	<i>D. gracillima</i>	<i>D. tokoro</i>	24.5	13.5	11	7	4.2	2.8	18.5	12.5	6	9.72 ^c	9.77 ^c
<i>D. quinqueloba</i>	<i>D. gracillima</i>	<i>D. tokoro</i>	11.5	9.5	2	7	4.2	2.8	18.5	12.5	6	1.09	2.12
<i>D. septemloba</i>	<i>D. nipponica</i>	<i>D. tenuipes</i>	7.5	4.5	3	22.5	12.5	10	18.8	12.8	6	7.50 ^c	7.53 ^b
<i>D. septemloba</i>	<i>D. quinqueloba</i>	<i>D. tenuipes</i>	8.5	5.5	3	10.5	9.5	1	17.8	11.8	6	0.21	2.07
<i>D. septemloba</i>	<i>D. gracillima</i>	<i>D. tenuipes</i>	9.5	5.5	4	7	4.2	2.8	16.8	11.8	5	0.38	0.39
<i>D. nipponica</i>	<i>D. quinqueloba</i>	<i>D. tenuipes</i>	21.5	11.5	10	8.5	7.5	1	20.8	14.8	6	5.63 ^b	8.21 ^b
<i>D. nipponica</i>	<i>D. gracillima</i>	<i>D. tenuipes</i>	24.5	13.5	11	7	4.2	2.8	17.8	12.8	5	9.72 ^c	9.77 ^c
<i>D. quinqueloba</i>	<i>D. gracillima</i>	<i>D. tenuipes</i>	11.5	9.5	2	7	4.2	2.8	17.8	12.8	5	1.09	2.12

^a mi, si, and vi indicate the number of total different sites, different transition sites, and different transversion sites from other two species in species i, respectively (i = 1, 2, and 3). ^b significant at 5% level; ^c significant at 1% level.

because the root was determined (Fig. 2C). When *D. gracillima*, *D. nipponica*, *D. quinqueloba*, and *D. septemloba* in the clade II were compared with each other, *D. tokoro* or *D. tenuipes* in the clade I was used as an outgroup species. When *D. tokoro* and *D. tenuipes* in the clade I were compared, *D. gracillima*, *D. nipponica*, *D. quinqueloba*, or *D. septemloba* in the clade II was used as an outgroup species. In these tests, each of heterozygous nucleotide was counted as a half substitution, and average number of differences between species were used for analysis. By both 1D and 2D methods, when *D. nipponica* was compared to *D. gracillima*, *D. quinqueloba* or *D. septemloba*, significant difference was detected at the 5% level. This result indicated that *D. nipponica* had a significantly faster substitution rate than the other three species in the clade II.

DISCUSSION

DNA polymorphism at the *Pgi* locus in *Dioscorea* species. Although *Dioscorea* species are all dioecious (completely outcrossing), the levels of polymorphism in the *Pgi* locus region of studied species were lower than other nuclear genes in other plants. It has been shown that outcrossing plant species generally have a larger amount of polymorphism than selfing species (Hamrick et al., 1979). The present result suggest that breeding system can not be the sole reason to determine the intraspecific variation in the *Dioscorea* species.

As reported for the entire coding region of the *Pgi* in *D. tokoro* by Terauchi et al. (1997), none of Tajima's test was significant for *D. tokoro*, *D. tenuipes*, *D. quinqueloba*, and *D. gracillima* for the investigated 1073-bp region. This result suggests that the pattern of nucleotide variation in the *Pgi* coding region of *D. tokoro*, *D. tenuipes*, *D. quinqueloba*, and *D. gracillima* does not deviate from neutrality. However, in this study, the sample size is the minimum for Tajima's test, and there is uncertainty of allelism in the comparison between *Pgi* sequences. In future, we may need to increase the number of sequences, and to clarify the ploidy and the number of the *Pgi* genes per the genome of the *Dioscorea* species.

Divergence at the *Pgi* between *Stenophora* species. The nucleotide substitutions per site in 1073 bp of the *Pgi* coding region between *Dioscorea* species investigated ranged from 1.4% to 4.0% (Table 4). These values are almost the same as those between sections of genus *Clarkia* (average difference = 3%: Gottlieb and Ford, 1996). In other words, nucleotide variation between *Dioscorea* species is as large as those between sections of other species. This result may suggest that morphological changes on which the classification of these *Dioscorea* species were based are slower than changes at the DNA level.

McDonald and Kreitman test (1991) conducted by Terauchi et al. (1997) showed that *D. tokoro* has an excess

of polymorphic replacement sites at the *Pgi* locus compared to fixed replacement sites. Contrary to the result of Terauchi et al. (1997), MK test in this study was not significant for any combinations of within and between species comparisons. This result suggests that the patterns of nucleotide substitution between polymorphism and divergence are similar with respect to amino acid changes in the coding region of the *Pgi* of investigated species. In this study, the sample size and the number of polymorphic sites are small. These may be related to the result that any of the MK tests were not significant.

The nucleotide positions of 335 (amino acid position 112) and 713 (position 238) determine three allozymes in *D. tokoro* (Terauchi et al., 1997). These two sites were G (Gly) and A (Gln) in all the studied species, including *D. tokoro* (DT37). This combination of G and A at these two sites corresponds to the b allozyme of PGI in *D. tokoro*. This result suggests that the b allozyme is the ancient form of the PGI in the *Dioscorea*, and that the other two a and c alleles were derived from b allele in *D. tokoro* as discussed in Terauchi et al. (1997).

Phylogenetic relationship between species in the section *Stenophora*. The phylogenetic trees (Fig. 2A and B) showed that investigated six species could be divided into two clades (I and II), supported by 100% of bootstrap probability. Of these two clades, the clade I consists of *D. tokoro* and *D. tenuipes*, and the clade II consists of *D. gracillima*, *D. nipponica*, *D. quinqueloba*, and *D. septemloba*. The clade I was also supported in the phylogenetic tree of amino acid sequence data (Fig. 2C) by a high bootstrap probability (91%). These results indicate that *D. tokoro* and *D. tenuipes* are monophyletic.

There seems to be some, but not clear, association between the clustering pattern in the phylogenetic tree and morphology and distribution of investigated species. *D. tokoro* and *D. tenuipes* in the clade I are morphologically similar to each other. Main differences in morphology between the two species are seed and stipule shapes. This morphological similarity may be related in the clustering of these two species. Of the four species in the clade II, *D. septemloba*, *D. quinqueloba*, and *D. nipponica* have lobed leaves. Although the clustering of these three species is not strong, this result suggests that species with lobed leaves may have been derived from the common ancestor with lobed leaves. Northern species (*D. gracillima*, *D. septemloba*, and *D. nipponica*) were included in the clade II. In addition, *D. quinqueloba* which distributes all over Japan is also included in this clade II. Therefore, this result suggests that nucleotide variation of the *Pgi* coding region does not reflect the difference in distribution pattern of species. The two species in the clade I are also distributed all over Japan. It is difficult to obtain a clear conclusion on the relationship between *Pgi* variation and geographic distribution from these results.

The faster evolutionary rate of the *Pgi* in *D. nipponica*. The results of the relative rate test (Table 5) indicated that substitution rate was significantly faster in *D. nipponica* lineage than the other three species in the clade II. The substitution rate is determined by mutation rate and fixation probability of mutations (Kimura, 1983; Li and Graur, 1991). If mutations are neutral, substitution rate is equal to mutation rate. Any deviation from neutrality introduces variation in the fixation probability mainly by the effect of natural selection.

A physiological study showed that *D. nipponica* was distinct in germination response at different temperatures (Okagami and Kawai, 1982; Okagami, 1986). *D. nipponica* maintains a high germination rate at high temperatures without dormancy break by chilling. On the other hand, the other species (*D. japonica*, *D. quinqueloba*, *D. septemloba*, *D. tenuipes*, and *D. tokoro*) have a low germination rate at high temperatures and requires chilling for dormancy break. If the PGI enzyme is related to adaptation at different temperatures (Riddoch, 1993), the different temperature response of *D. nipponica* may be related to the significantly faster substitution rate in the *Pgi* locus of this species. At this moment, we do not have any clear explanation for the significantly faster evolutionary rate in the lineage of *D. nipponica*.

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