Nucleotide Sequence Variations in the *Adh* in a Medicinal Species, *Dioscorea tokoro* (Dioscoreaceae)

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A medicinal species, *Dioscorea tokoro* (Dioscoreaceae), distributes in temperate eastern Asia including the entire Japan Islands. To determine the colonizing history of this species into Japan, we investigated the geographic pattern of its nucleotide sequence variations in Japan. To reveal the genetic relationships between the species, forty-five *Dioscorea* species were reconstructed molecular phylogenetic tree using the *rbcL* and *matK* sequences, indicating that *D. tokoro* is the sister to *D. tenuipes*. Moreover, the result of network analysis of the *Adh* sequences using thirty individuals of *D. tokoro* indicates that there are two genetic groups in Japan, suggesting that this species had independently colonized into Japan at least twice.

Keywords : chloroplast DNA, *Dioscorea*, molecular analysis, nuclear DNA

INTRODUCTION

The phylogenetic relationship that characterizes commercially important crops and their wild relatives is a matter of considerable concern, as it is generally agreed that access to genetic diversity has been and remains important in maintaining and increasing agricultural productivity. The genus *Dioscorea* L. is involved in widely cultivated crops such as *D. alata* L., *D. batatas* Decne., and *D. japonicum* Thunb. and various important characteristics, including good nutritional and pharmaceutical properties (Mwiringi et al., 2009). Despite their importance, breeding and selection of crop genotypes with improved traits is currently inhibited by the lack of adequately characterized native genotypes at the morphological and molecular level (Dansi et al., 1999). This is due to the fact that the distribution of genotypes are not well documented, which constraints the efficient conservation of these genetic resources thereby limiting their use in breeding programs.

Colonization and distribution of plants offer useful models for studying rapid range expansion in novel environments, which can imply pre-adaptation, phenotypic plasticity or adaptation. When considered in colonizing and distribution pattern under novel environment, the structuring of genetic variation is a product of historical relationships between populations of a given useful plant. In this case, phylogeographical analysis provides powerful methods with which to tease apart the relevant processes that lead to population structure in species (Avise et al., 1987; Avise, 2000). By taking into account the genealogical relationships between haplotypes as well as their geographical distribution, phylogeographical methods can potentially determine the historical processes that

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shape current patterns of variation.

The number of phylogenetic studies based on molecular data has grown enormously in recent years, and most of the recent studies are concerned with closely related species or variation within species. In particular, the use of molecular markers has considerably improved our knowledge about how past events shape the genetic diversity within a species (Avice et al., 1987; Avice, 2000). Moreover, in conjunction with advances in molecular techniques and analytical methods on haplotype data, nuclear gene regions provide enough intraspecific variation and have been successfully used in addressing various population and evolutionary questions on plant species including crops and their wild relatives (Olsen and Schaal, 1999; Caicedo and Schaal, 2004; Londo et al., 2006; Muller et al., 2006). For example, the application of phylogenetic analysis is emerging as an important and practical tool for the study of economically important species such as rice, maize, sunflower and cassava, and their relatives (Beckler and Holtsford, 1996; Olsen and Schaal, 1999; Olsen, 2002; Olsen and Purugganan, 2002; Liu et al., 2003; Vigouroux et al., 2003; Harter et al., 2004). The ancestors and close relatives of a given species have repeatedly provided sources of useful traits to genetically impoverished crops. Moreover, conscious efforts to search for desirable traits in plants have been underway for the past century, and in recent decades species with desirable traits have come to be regarded as important biological resources in need of conservation (Tanksley and McCouch, 1997). The population structure and history of a given species is thus an important research focus, because this knowledge is needed to design concerted efforts for conservation of the species and to understand the evolutionary processes leading to genetic diversity. Recent decades have witnessed the widespread use of DNA sequences in phylogeographic studies of various plant species (e.g., Fukuda et al., 2001; Petit et al., 2005). In Japan, molecular approaches have been used to analyze the intraspecific genetic variation of a number of different plant species (e.g., *Alpinia japonica*, *Arachnioides sporadosora*, *A. aristata*, *Daphne kiusiana*, *Elaeocarpus sylvestris* var. *ellipticus*, *Prunus zippeliana*, Aoki et al., 2004). These markers are mostly neutral with relative high mutation rates, and, in association with the geological history, provide information to estimate the extent of seed dispersal and track migration routes (Ferris et al., 1993; Dumolin-Lapegue et al., 1997; Schaal and Olsen, 1999; Fujii et al., 2002).

Dioscorea tokoro Makino is a dioecious, diploid species that belongs to Dioscoreaceae. This species has a rhizophore which is either branched in a ginger-like shape or in irregular masses with fibrous roots and has been frequently used to cure diarrhea, cough, leukorrhea and frequency of urination and arthritis (Zhong et al., 2002). Moreover, the rhizophore of this species has been reported to decrease damage in renal tubules, inflammation in the central vein and necrosis in the liver because of its anti-inflammatory action (Lee et al., 2002). It is mainly distributed in temperate eastern Asia including the entire Japan Islands. Although this species widely distributed in Japan, its patterns of range expansion remains unclear. Such information of nucleotide variations is indispensable for exploitation and utilization of wild resources and genetic improvement of this species and is vitally important for developing conservation strategies for this economically important species. However, phylogenetic studies of species of Dioscoreaceae had not been made so far, therefore we can not select appropriate outgroup taxa to use the phylogeographic study of *D. tokoro*. The outgroup taxa are important for assessing plesiomorphic or apomorphic states for each characteristic and for interpreting the evolutionary history of this species. Wilkin et al. (2005) constructed the phylogenetic tree in the genus *Dioscorea* without *D. tokoro*. To reveal the phylogenetic position of *D. tokoro*, we reconstructed the phylogenetic tree using chloroplast DNA (cpDNA) sequences. Moreover, we describe the DNA polymorphisms of the alcohol dehydrogenase (*Adh*) of nuclear DNA (nrDNA) of this species, which gene has been the subject of population and evolutionary genetic survey in many plant species (*Arabidopsis thaliana*: Hanfstingl et al., 1994; Innan et al., 1996; *Paeonia* species: Sang et al., 1997; *Hordeum vulgare* ssp. *spontaneum*: Cummings and Clegg, 1998; *Miscanthus sinensis* and *M. condensatus*: Chiang et al., 2003; *Oryza rufipogon*: Yoshida et

al., 2004; Yoshida and Miyashita, 2005; *Calystegia soldanella*: Ohsako and Matsuoka, 2008). Terauchi et al. (1997) reported the nucleotide sequence variations in the *Adh* in *D. tokoro* using the populations from the limited regions. To enhance our knowledge, we consider colonization and distribution of *D. tokoro* using cpDNA and the *Adh* of nrDNA.

MATERIALS AND METHODS

Plant materials

To conduct molecular phylogenetic analysis using the ribulose 1,5-bisphosphate carboxylase large subunit (*rbcL*) and the maturase K (*matK*) of cpDNA, 45 samples of *Dioscorea* species were examined in this study (Table 1). Of these sequences, 37 *Dioscorea* species were collected in the DDBJ/EMBL/GenBank International DNA databases. *Stenomeris borneensis*, *Tacca integrifolia* and *T. leontopetaloides* were selected as outgroups on the basis of phylogenetic analyses of this genus. In addition, thirty samples of *D. tokoro* were analyzed using the *Adh* sequences of nrDNA (Table 2). Vouchers for the species sampled in this study have been deposited in the Herbarium, Graduate School of Science, Tohoku University (TUS) and the Herbarium of Makino Botanical Garden, Kochi (MBK).

Molecular analysis

Total DNAs were isolated from dried leaves with a Plant Genomic DNA Mini Kit (VIOGENE, Sunnyvale, CA, USA), according to the manufacturer's protocols. The PCR reaction mix contained approximately 100 to 200 ng of total DNA, 1 μ M of each primer, 200 mM of each deoxynucleotide, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM of MgCl₂, and 1.25 units of *Taq* polymerase. Double-stranded DNA was amplified, after incubation at 94°C for 2 min, by thirty cycles of incubation at 94°C for 1 min, 48°C for 2 min and 60°C for 2 min, with final extension at 72°C for 15 min. We amplified the *rbcL*, *matK* and *Adh* with primers designed by Morgan and Soltis (1993), Johnson and Soltis (1994) and Sang et al. (1997), respectively. For some samples, we used the following primers designed in this study (matK1DF: 5'-CCC AAG ARA TGY YCC YWR YT-3', matK1DR: 5'-TGY AAA GAR GAA ACA TCT TG-3', matK2DF: 5'-CAA GAT ATT TAY TCT TTR CA-3', matK2DR: 5'-TCC YTT TGA CAC CAK AAT TG-3', matK3DF: 5'-CAA TTM TGG TGT CAA ARG GA-3', matK3DR: 5'-GGA TAG AAK AHR ARA CCC CT-3'). After amplification, reaction mixtures were subjected to electrophoresis in 1.0% low-melting-temperature agarose gels for separation of specific amplified products. We sequenced the purified PCR products using a BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, PRISM DNA Sequencing kit, Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100-Avant Genetic Analyzer according to the manufacturer's instructions. The primers used for sequencing were the same as those used for amplification.

To construct a phylogenetic tree based on the *rbcL* and *matK* sequences of *Dioscorea* and its allied species, the amplified regions (minus the length of the primers) were aligned using Clustal W (Thompson et al., 1994) and were improved manually using MEGA 4 (Tamura et al., 2007). In the phylogenetic analyses, the most-appropriate model of DNA substitution was chosen using hierarchical likelihood ratio tests with PAUP*4.08b (Swofford, 2001) and Modeltest 3.0 (Posada and Crandall, 1998). TVM model (Rodríguez et al., 1990) with invariable sites of 0.4403 and a gamma shape parameter of 1.0147 (TVM+I+G) was chosen (Base frequencies: A, 0.2900; C, 0.1782; G, 0.1948; and T, 0.3370. Substitution rates: A-C, 1.2199; A-G, 3.1068; A-T, 0.3180; C-G, 0.9533; C-T, 3.1068; G-T, 1.0000). The maximum likelihood (ML) analysis was performed using PAUP 4.0b10 with heuristic searches, starting trees obtained by neighbor joining (NJ), and TBR branch swapping. Phylogenetic relationships were analyzed using the neighbor-joining (NJ) method with PAUP*4.08b (Swofford, 2001). The NJ analyses were performed using MEGA 4 with Kimura's two-parameter model. NJ and ML trees were assessed using non-parametric bootstrapping of 1000

Table 1 Accession numbers of *matK* and *rbcl* using phylogenetic analysis of *Dioscorea* and outgroup taxa.

Species	<i>matK</i>		<i>rbcl</i>	
	Acc. No.	Reference	Acc. No.	Reference
<i>Dioscorea alata</i>	AB040208	Tamura (2000)	AY667098	Wilkin et al. (2005)
<i>D. antaly</i>	AY956476	Wilkin et al. (2005)	AY667100	Wilkin et al. (2005)
<i>D. arachidna</i>	AY956478	Wilkin et al. (2005)	AF307468	Caddick et al. (Unpubl.)
<i>D. arcuatinervis</i>	AY956479	Wilkin et al. (2005)	AY667102	Wilkin et al. (2005)
<i>D. batatas</i>	AB624369	this study	AB624377	this study
<i>D. bemarivensis</i>	AY956480	Wilkin et al. (2005)	AY667103	Wilkin et al. (2005)
<i>D. birmanica</i>	AY956481	Wilkin et al. (2005)	AY667104	Wilkin et al. (2005)
<i>D. brachybotrya</i>	AY956482	Wilkin et al. (2005)	AF307469	Caddick et al. (Unpubl.)
<i>D. buchananii</i>	AY956487	Wilkin et al. (2005)	AY904790	Wilkin et al. (2005)
<i>D. bulbifera</i>	AY956488	Wilkin et al. (2005)	AY904791	Wilkin et al. (2005)
<i>D. c.f. apurimaccensis</i>	AY956477	Wilkin et al. (2005)	AY667101	Wilkin et al. (2005)
<i>D. collettii</i>	AB624368	this study	AB624376	this study
<i>D. communis</i>	AY956490	Wilkin et al. (2005)	AF307474	Caddick et al. (Unpubl.)
<i>D. cordata</i>	AY973835	Wilkin et al. (2005)	AF307472	Caddick et al. (Unpubl.)
<i>D. daunea</i>	AY956493	Wilkin et al. (2005)	AY904793	Wilkin et al. (2005)
<i>D. decipiens</i>	AY956494	Wilkin et al. (2005)	AF307454	Caddick et al. (Unpubl.)
<i>D. deltoidea</i>	AB624366	this study	AB624372	this study
<i>D. dumetorum</i>	AY956495	Wilkin et al. (2005)	AF307464	Caddick et al. (Unpubl.)
<i>D. elephantipes</i>	AY956496	Wilkin et al. (2005)	AF307461	Caddick et al. (Unpubl.)
<i>D. fandra</i>	AY956498	Wilkin et al. (2005)	AY904795	Wilkin et al. (2005)
<i>D. galeottiana</i>	AY956499	Wilkin et al. (2005)	AY904796	Wilkin et al. (2005)
<i>D. gillettii</i>	AY956500	Wilkin et al. (2005)	AY904797	Wilkin et al. (2005)
<i>D. glabra</i>	AY956501	Wilkin et al. (2005)	AF307456	Caddick et al. (Unpubl.)
<i>D. gracillima</i>	AB624364	this study	AB624371	this study
<i>D. hamiltonii</i>	AY957687	Wilkin et al. (2005)	AF307465	Caddick et al. (2000)
<i>D. hexagona</i>	AY957588	Wilkin et al. (2005)	AY904798	Wilkin et al. (2005)
<i>D. hispida</i>	AY957589	Wilkin et al. (2005)	AF307463	Caddick et al. (2000)
<i>D. inopinata</i>	AY957590	Wilkin et al. (2005)	AY904799	Wilkin et al. (2005)
<i>D. japonica</i>	AB624362	this study	AB624374	this study
<i>D. lanata</i>	AY957593	Wilkin et al. (2005)	AF307458	Caddick et al. (Unpubl.)
<i>D. longirhiza</i>	AY957595	Wilkin et al. (2005)	AF307473	Caddick et al. (Unpubl.)
<i>D. maciba</i>	AY957596	Wilkin et al. (2005)	AY904800	Wilkin et al. (2005)
<i>D. mcvaughii</i>	AY957597	Wilkin et al. (2005)	AF307460	Caddick et al. (Unpubl.)
<i>D. membranacea</i>	AY957598	Wilkin et al. (2005)	AF307467	Caddick et al. (Unpubl.)
<i>D. oryzetorum</i>	AY972482	Wilkin et al. (2005)	AY904801	Wilkin et al. (2005)
<i>D. pentaphylla</i>	AY972483	Wilkin et al. (2005)	AF307470	Caddick et al. (Unpubl.)
<i>D. rockii</i>	AY972488	Wilkin et al. (2005)	AY939882	Wilkin et al. (2005)
<i>D. sansibarensis</i>	AY972489	Wilkin et al. (2005)	AY939883	Wilkin et al. (2005)
<i>D. schimperiana</i>	AY972490	Wilkin et al. (2005)	AF307466	Caddick et al. (Unpubl.)
<i>D. septemloba</i>	AB624365	this study	AB624370	this study
<i>D. soso</i>	AY972491	Wilkin et al. (2005)	AY939884	Wilkin et al. (2005)
<i>D. tenuipes</i>	AB624364	this study	AB624375	this study
<i>D. tokoro</i>	AB624367	this study	AB624373	this study
<i>D. wallichii</i>	AY973830	Wilkin et al. (2005)	AY939888	Wilkin et al. (2005)
<i>D. zingiberensis</i>	AY973831	Wilkin et al. (2005)	AY939889	Wilkin et al. (2005)
Outgroup				
<i>Stenomeris borneensis</i>	AY973836	Wilkin et al. (2005)	AF307475	Caddick et al. (Unpubl.)
<i>Tacca integrifolia</i>	AY973838	Wilkin et al. (2005)	AF307478	Caddick et al. (Unpubl.)
<i>T. leontopetaloides</i>	AY973839	Wilkin et al. (2005)	AF307480	Caddick et al. (Unpubl.)

pseudoreplicates (Felsenstein and Kishino, 1993) to examine the robustness of the clades and their phylogenetic relationships. In addition, a network tree of the *Adh* was constructed with NETWORK 1.4.1.2 (Fluxus Technology Ltd., Suffolk, UK) using the median joining method

Table 2 Samples using the network analysis of *Dioscorea tokoro* and its allied species based on *Adh* of nrDNA.

No. Species	Symbol	Locality	Date	Collector	Herbarium No.
1 <i>Dioscorea tokoro</i>	●	Aomori city, Aomori Pref.	3 Sep. 1998	Yonekura K.	TUS 4530
2 <i>D. tokoro</i>	●	Mt. Minaminata, Arakawa town, Aomori city, Aomori Pref.	8 Aug. 2000	Yonekura K.	TUS 746
3 <i>D. tokoro</i>	●	Komagamine, Ishimaki city, Miyagi Pref.	22 Jul. 1995	Hayasaka E.	
4 <i>D. tokoro</i>	●	Komagamine, Ishimaki city, Miyagi Pref.	4 Aug. 1995	Hayasaka E.	TUS 93462
5 <i>D. tokoro</i>	●	Kawauchi Kameoka town, Aoba-ku, Sendai city, Miyagi Pref.	2 Aug. 1993	Yonekura K.	TUS 3030
6 <i>D. tokoro</i>	●	Mt. Ganto, Kawasaki city, Miyagi Pref.	27 Sep. 1993	Azuma T. et al.	
7 <i>D. tokoro</i>	●	Yamagata city, Yamagata Pref.	10 Aug. 2000	Hayasaka E. et al.	TUS 10672
8 <i>D. tokoro</i>	●	Kariyasu, Yonezawa city, Yamagata Pref.	22 Sep. 2009	Yokoyama J.	
9 <i>D. tokoro</i>	●	Shima, Nakanojo-Machi, Agatsuma-Gun, Gunma Pref.	27 Aug. 2003	Yonekura K.	
10 <i>D. tokoro</i>	●	Ueda city, Nagano Pref.	7 Aug. 1996	Ikeda T.	
11 <i>D. tokoro</i>	●	Mt. Ryozen, Inukami-gun, Shiga Pref.	8 Oct. 1961	Tamura M.	
12 <i>D. tokoro</i>	●	Sogabe town, Kameoka city, Kyoto Pref.	27 Jul. 2003	Inumochi M.	
13 <i>D. tokoro</i>	●	Ashizuri cape, Ashizuri city, Kochi Pref.	25 Jul. 2004	Tashio M. et al.	
14 <i>D. tokoro</i>	●	Ootani, Susaki city, Kochi Pref.	29 Sep. 2001	Kuroiwa N. et al.	TUS 22205
15 <i>D. tokoro</i>	●	Machiagami, Iduhara-machi, Tsushima city, Nagasaki Pref.	17 Sep. 2009	Hayakawa H.	
16 <i>D. tokoro</i>	■	Achi vilege, Shimoina-gun, Nagano Pref.	3 Oct. 2003	Asano K. & Asano H.	TUS 3575
17 <i>D. tokoro</i>	■	Mt. Ikoma, Higashiosaka City, Osaka Pref.	1 Oct. 2002	Kanno M.	
18 <i>D. tokoro</i>	■	Nakafuji, Tanto town, Toyooka city, Hyogo Pref.	6 Oct. 1996	Fukuoka N. & Kurosaki N.	
19 <i>D. tokoro</i>	■	Kokenawa, Kamigori-cho, Akou-gun, Hyogo Pref.	28 Jun. 1999	Yauchi M.	
20 <i>D. tokoro</i>	■	Akiyoshi, shuho-cho, Mine-shi, Yamaguchi Pref.	6 Aug. 1995	Oka K.	
21 <i>D. tokoro</i>	■	Kawauchi, Toyo town, Aki-gun, Kochi Pref.	10 Aug. 2003	Kogami Y. et al.	
22 <i>D. tokoro</i>	■	Ananai, Tosayamada town, Kami city, Kochi Pref.	27 May. 2009	Fukuda T.	
23 <i>D. tokoro</i>	■	Nagase, Kahoku town, Kochi Pref.	05 Jul. 2003	Fukuhashi H.	MBK 0061946
24 <i>D. tokoro</i>	■	Yoshidome, Munakata city, Fukuoka Pref.	11 Sep. 1995	Watanabe S.	
25 <i>D. tokoro</i>	■	Mt. Taryama, Kuguruki-cho, Sasebo city, Nagasaki Pref.	1 Sep. 1993	Yonekura K.	
26 <i>D. tokoro</i>	■	Yabakei, Nakatsu city, Oita Pref.	29 Sep. 2009	Hayakawa H.	
27 <i>D. tokoro</i>	▲	Kitauranyudozaki, Ogashi city, Akita Pref.	4 Sep. 2007	Horii Y.	TUS 35052
28 <i>D. tokoro</i>	▲	Tiyodani, Ikeda-cho, Imadate-gun, Fukui Pref.	1 Aug. 1998	Kurosaki N.	TUS 21086
29 <i>D. tokoro</i>	▲	Yusuhara-cho, Takaoka-gun, Kochi Pref.	3 Aug. 2003	Hosokawa K. et al.	TUS 58886
30 <i>D. tokoro</i>	▲	Mt. Takakura, Kagoshima Pref.	12 Aug. 1942	Furusawa I.	TUS 121942
Outgroup					
31 <i>D. gracillima</i>		Narukawa, Ikegawa town, Kochi Pref.	16 May 2007	Kobayashi S.	MBK 0190875

TUS: Herbarium of Graduate School of Science, Tohoku University

MBK: Herbarium of Makino Botanical Garden, Kochi.

(Bandelt et al., 1999).

RESULTS

To construct the molecular phylogenetic tree of *Dioscorea* and its allied species, we determined sequences of the *rbcL* and *matK* of *Dioscorea* species and three outgroups of *Stenomeris borneensis*, *Tacca integrifolia* and *T. leontopetaloides*. The length of the *rbcL* and *matK* was 1277 bp and 1458 bp, respectively (without InDels). The result of phylogenetic relationship indicated that *Dioscorea* species was consisted of a monophyletic group with high bootstrap value (Figs. 1

Fig. 1 Phylogenetic relationships of *Dioscorea* species based on NJ analysis of *rbcL* and *matK* sequences of cpDNA. Bootstrap values are given above and below branch of clades (more than 50%). Sample numbers correspond to Table 1.

and 2). Moreover, *Dioscorea tokoro* was concluded in a monophyly with *D. collettii*, *D. deltoidea*, *D. gracillima*, *D. membranacea*, *D. rockii*, *D. septemloba*, *D. tenuipes* and *D. zingiberensis* and this monophyletic group was placed at the most basal position of Dioscoreaceae based on both NJ and ML trees. This result indicates that most closely related species of *D. tokoro* is *D. tenuipes* (BS value: 98%). Some closely related species of *D. tokoro* were clarified from the phylogenetic analysis, therefore, *D. gracillima* and *D. septemloba* were selected as appropriate outgroups of phylogeographical analysis of this species.

Fig. 2 Phylogenetic relationships of *Dioscorea* species based on ML analysis of *rbcL* and *matK* sequences of cpDNA. Bootstrap values are given above branch of clades (more than 50%). Sample numbers correspond to Table 1.

To construct a network relationship of *D. tokoro*, we determined the *Adh* sequences from 30 samples of *Dioscorea tokoro* with one outgroup (AB619606-17). The lengths of *Adh* sequences were 421 bp (including InDels) in *D. tokoro*. Two groups in the *Adh* were recognized in this species, namely Type 1 and Type 2 (Fig. 3), depend on the mutation site in exon 4 with 3 related species, *D. gracillima* (AB619617), *D. tenuipes* (AB006002), and *D. nipponica* (data not shown). Type 1 was consisted of 15 samples from the Kyushu district (Nagasaki Prefecture), the Shikoku district (Kochi Prefecture), the Kinki district (Kyoto and Shiga prefectures), the Chubu district (Nagano Prefecture), the Kanto district (Gunma Prefecture), and the Tohoku district (Miyagi, Yamagata, and Aomori prefectures) (Fig. 4). In contrast, Type 2 was concluded in 11 samples from the Kyushu district (Oita, Fukuoka, and Nagasaki prefectures), the Chugoku district

Fig. 3 Parsimony network of *Dioscorea tokoro* based on *Adh* sequences of nrDNA. Each group is boxed by dashed line. Numbers in the circles indicate the samples corresponding to Table 2. Closed circles among haplotypes represent a mutational step.

Fig. 4 Genetic distribution of *Dioscorea tokoro* used in this study. Black circles indicate Type 1, black squares are Type 2 and black triangles are heterozygous for Type 1 and Type 2. Numbers correspond to the sample number in Table 2.

(Yamaguchi Prefecture), the Shikoku district (Kochi Prefecture), the Kinki district (Hyogo, Shiga, and Osaka Prefectures) and the Chubu district (Nagano Prefecture) (Fig. 4). In addition, heterozygous for Type 1 and Type 2 was found in four samples from the Kyushu district (Kagoshima Prefecture), the Hokuriku district (Fukui Prefecture), the Shikoku district (Kochi Prefecture) and the Tohoku district (Akita Prefecture) (Fig. 4).

DISCUSSION

In this study, we investigated the geographical structure of nucleotide sequence variations in *Dioscorea tokoro*. To clarify phylogenetic relationships within *D. tokoro*, therefore, we previously determined nucleotide sequences of ten regions of cpDNA; *trnG* (UCC) intron: 313 bp, *atpF* intron: 313 bp, *psbC-trnS* (UGA): 198 bp, *trnG* (GCC)-*trnM* (CAU): 137 bp, *trnV* (UAG)-*trnM* (CAU): 229 bp, *trnW* (CCA)-*trnP* (UGG): 273 bp, *petB* intron: 332 bp, *rpl16* intron: 282 bp, *trnT-trnL*: 815 bp and *trnL* intron: 565 bp (Accession Nos. AB557679 - AB557688); which regions were the high substitution rates and well determined for intraspecific phylogeny (Taberlet et al., 1991; Nishizawa and Watano, 2000). However no apomorphic characters were found among individuals of *D. tokoro*. Therefore, it is likely that this species has undergone a recent, rapid radiation because of no accumulation of nucleotide mutations. Indeed, some episodes of recent rapid radiation in plants have been documented in oceanic archipelagos (Baldwin and Sanderson, 1998; García-Maroto et al., 2009), megadiverse floristic hotspots such as the tropics (Richardson et al., 2001; Kay et al., 2005; Hughes and Eastwood, 2006), the Cape of South Africa (Verboom et al., 2003; Klak et al., 2004) and East Asia (Yamashiro et al., 2004; Fukuda et al., 2005a). To confirm the scenario of *D. tokoro*, therefore, we additionally determined the internal transcribed spacer (ITS) region of nrDNA using previous published primers by White et al. (1990). In general, the usefulness of the ITS region as a molecular marker for the indirect estimation of infraspecies relationships depends on the level of phylogenetic resolution (Baldwin et al., 1995), and Sang et al. (1995) suggested that the ITS region had faster divergence rates and subsequently higher phylogenetic resolution than regions of cpDNA. Therefore, the ITS region had been reported by Beckler and Holtsford (1996) who used the ITS phylogenetic tree to reveal the recent derivation of maize from teosinte, and by Dick et al. (2003), who concluded on the basis of various ITS phylogenetic trees that the ITS region is a powerful tool for resolving historical relationships among populations of widespread plants. Although we determined the 538 bp of ITS region of *D. tokoro*, there are no variations among individuals of this species (Accession No. AB557689). As a result, we concluded that the ITS region is powerless for phylogenetic studies within this species, and this result supports that this species had expanded its range to these areas of Japan in more recent years.

The *Adh* gene were isolated from various plant species (Fukuda et al., 2005b) and reported from *Dioscorea tokoro* (Terauchi et al., 1997). We found in current study that the *Adh* gene possesses a relatively high level of nucleotide variation. This region, therefore, provides sufficient phylogenetic resolution at a population level within *D. tokoro*. Moreover, it is interesting that two groups, Type 1 and Type 2, of this species were involved in Japan, but it is unclear whether or not these groups were originated in Japan. Our results suggest that there had been patterns of range expansion of Japan at least twice because there are relatively genetic differentiations between two groups and the center of diversification of the genus *Dioscorea* is the tropical and the subtropical areas of the world (Heywood, 1978). The genetic differentiation between them may be accumulated in the tropical and the subtropical areas, therefore, we hypothesize that *D. tokoro* is originated and differentiated in the tropical and the subtropical areas and colonized independently into Japan. Two sequencing substitutions could be found in the *Adh* gene of *D. tokoro* although there is no variation in cpDNA and ITS sequences, suggesting that two different lineages of the *Adh* gene had been experienced rapid expansion of its distribution after independent colonization into Japan. To

support our hypothesis, this species of the tropical and the subtropical areas was analyzed in the future.

In Japan, a lofty backbone range that basically runs along the axis of Honshu or Shikoku and Kyushu and divides the island into two areas. Therefore, some local topographical barriers in the central zone may have blocked or limited the migration to the opposite coasts, with considerable effects on the genetic structure of plant species. For example, Fujii et al. (2002) and Okura and Harada (2002) found that a phylogenetic tree of *Fagus crenata* (Fagaceae) was roughly divided into two clades that were composed of populations growing on the Pacific Oceanic side and the Japan Sea side by the axis of Honshu, respectively. Moreover, the north and south aspects of the Shikoku and Kyushu mountain range experience different climatic conditions and different vegetation types have developed accordingly (Miyawaki, 1987). In our study, however, no significant genetic differences could be detected between *Dioscorea tokoro* individuals (Fig. 4). This may not reflect geographical patterns of genetic distribution of *D. tokoro*. Both groups of this species have similar distribution in the southwestern area of Japan, and so that the distribution may have been expanded various areas of Japan through the same route. Of them, Type 1 was found in broad areas but Type 2 was restricted in the southern and the central areas of Japan. From this result, we suggest that Type 2 could not be expanded into the northern area of Japan. However, the heterozygous of them could be detected in the individual of the Tohoku district (Akita Prefecture), which is located at the northern area of Japan. It is unknown whether the individuals of heterogeneity colonized into the Tohoku district or there are any crossings between the Type 1 and the undetectable Type 2 in this region from our study caused by the limited samples, especially the northern area of Japan. Further sampling and analyses of this area allow us to answer this question.

The interest in trying to link geographic distribution and evolution of the *Adh* gene with organism evolution rests on the wide use of such markers for phylogeny as well as on the increasing number of cases documenting complicated evolution in plants. The *Adh* gene analyzed in this work provides a robust phylogeographic hypothesis for the evolution of *Dioscorea tokoro*. This hypothesis, based on molecular information, is a timely contribution that allows unbiased interpretation of evolutionary history. Additional molecular markers may lend support to this scenario, and multiple markers should be surveyed within this species to find any variation. This work will help to reinterpret existing *Adh* data sets and facilitate the interpretation of new ones.

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