HOKKAIDO UNIVERSITY

Title	Ploidy distribution and DNA content variations of Lonicera caerulea (Caprifoliaceae) in Japan
Author(s)	Miyashita, Tomomi; Araki, Hajime; Hoshino, Yoichiro
Citation	Journal of Plant Research, 124(1), 1-9 https://doi.org/10.1007/s10265-010-0341-6
Issue Date	2011
Doc URL	http://hdl.handle.net/2115/44725
Rights	The original publication is available at www.springerllink.com.
Type	article (author version)
File Information	hoshino.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Corresponding author: Yoichiro Hoshino

Address: Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita–ku, Sapporo 060–0811, Japan

E-mail: hoshino@fsc.hokudai.ac.jp

phone number: +81-11-706-2857

Fax number: +81-11-706-2857

Whether the corresponding author is a member or non-member of the Botanical Society of Japan: member

Subject area: (1) Taxonomy/Phylogenetics/ Evolutionary, Biology

Number of tables, black-and-white figures, and color figures:

Three tables, 6 black-and-white figures

Title: Ploidy distribution and DNA content variations of *Lonicera caerulea* (Caprifoliaceae) in Japan

Authors: Tomomi Miyashita[1], Hajime Araki[1,2] and Yoichiro Hoshino[1,2]

Affiliations: [1]Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, JAPAN

[2]Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, JAPAN

Abstract

Ploidy level and geographical distribution were investigated in Japanese *Lonicera caerulea* L. Flow cytometric analysis revealed the presence of DNA diploid and DNA tetraploid plants in Japan. Chromosome observation confirmed that diploid and tetraploid plants showed $2n = 2x = 18$ and $2n = 4x = 36$, respectively. The DNA diploid populations were found only in lowland mires, Betsukai, Bekanbeushi, Kushiro and Kiritappu located in eastern Hokkaido. On the other hand, DNA tetraploid populations were distributed in a wide area of Hokkaido, and mainland of Japan. The habitats of DNA tetraploid plants were lowland to alpine region. The DNA content measurement with flow cytometry revealed significant differences in the relative DNA contents among DNA tetraploid populations. The relative DNA content within DNA tetraploid populations varied 1.157-fold at maximum, and might correlate with altitude indicating that DNA contents were smaller as altitude increases. The wide area of distribution in various environments of DNA tetraploid plants suggested the adaptability of the tetraploid plants. Although diploid and tetraploid populations were found, no triploid was detected, indicating crossing difficulty between diploid and tetraploid as confirmed by crossing experiment.

Key words: chromosome number; DNA ploidy level; flow cytometry; genome size variation; geographical distribution; *Lonicera caerulea*

Introduction

Polyploidy has played a key role in plant evolution. Generally, polyploids differ from their progenitors in morphological, ecological, physiological and/or cytological characteristics. Consequently, polyploid may show exploitation of a new niche and reproductive isolation (reviewed by Ramsey and Schemske 2002). Thus, investigation of ploidy distribution is important issue to understand evolutionary adaptation and species specification in polyploid plant.

The genus *Lonicera* belongs to the family Caprifoliaceae, and comprises more than 200 species (Naugžemys et al. 2007). Some species in this genus are used as ornamental and edible plants. One of them, namely, *Lonicera caerulea* L., is generically known as blue honeysuckle; it is a shrub with edible fruits. *L. caerulea* is distributed in northern Eurasia, Japan and North America. The fruits of wild *L. caerulea* are traditionally used in Japan, Russia, and northern China (Anetai et al. 1996; Thompson and Chaovanalikit 2003). It has been cultivated as a berry crop, particularly in Russia and Japan, because the fruits are rich in nutrients such as polyphenolics and anthocyanins (Chaovanalikit et al. 2004; Terahara et al. 1993), and its cultivation is economical.

In natural populations of *L. caerulea*, diploid ($2n = 2x = 18$) and tetraploid ($2n = 4x = 36$) have been observed in Europe to Asia (Ammal and Saunders 1952). Limited information about ploidy levels of *L. caerulea* has been noted in Japanese populations. Solovyeva and Plehanova (2003) and Naugžemys et al. (2007) indicated taxonomic obscurity in *Lonicera* subsection *caeruleae* Rehder. So far, although different scientific names were used in published manuscripts, following findings were obtained. Hara (1983) investigated Caprifoliaceae of Japan and described tetraploid ($2n = 36$) in *L.*

caerulea subsp. *edulis* (Turcz.) Hultén var. *edulis*. Plekhanova et al. (1992) examined chromosome numbers and distribution area of *Lonicera* subsection *caeruleae* in far east of Asia to northern regions of Eurasia. They described *L. emphyllocalyx* Maxim. of Hokkaido, Japan as tetraploid ($2n = 36$). Plekhanova (2000) referred large population of *L. caerulea* L. ($2n = 36$) existing on Hokkaido, Japan. Thus, only tetraploid plants have been reported in Japan. However, little is known about the geographical distribution and ploidy of *L. caerulea* in Japan.

In the present study, we determined DNA ploidy levels by flow cytometry in the population of Japanese *L. caerulea*, and their chromosome numbers were observed. In addition, unexpected DNA content variations were found by flow cytometry in DNA tetraploid plants. Furthermore, geographical patterns of ploidy distribution and DNA content variations were discussed.

Materials and Methods

Plant materials

The sources of plant materials (*Lonicera caerulea* L.) used in this study are shown in Table 1. *Lonicera caerulea* is native to Japan and is called as ‘blue honeysuckle’ or locally ‘Haskap’. In Japan, this species is distributed on mountain areas in mainland, and in Hokkaido. Basic chromosome number is $x = 9$.

In order to evaluate ploidy levels of Japanese *L. caerulea*, we have collected wild species in Hokkaido. Sampling locations of natural populations could be entirely covered Hokkaido. One location was added from mainland of Japan. For this study, 450 wild *L. caerulea* individual plants from 21 populations were collected (Table 1, Fig. 1) in Japan between 2008 and 2009. Plants from natural populations of *L. caerulea* in the

eight locations in Hokkaido, namely, Betsukai, Bihoro Pass, Mt. Daisetsu, Mt. Piyashiri, Mt. Yokotsudake, Taiki, Kiritappu mire, and Yufutsu mire, were provided by the Hokkaido Forestry Research Institute (HFRI). The plants from Senjogahara mire in Tochigi Prefecture on the mainland were obtained from collections at Nikko Botanical Garden (NBG), Graduate School of Science, The University of Tokyo. Two plants from Kiritappu mire were obtained from collections at Hokkaido University. We also collected wild *L. caerulea* plants from Betsukai; Mt. Yokotsudake; Mt. Eboshidake; the Bekanbeushi, Kiritappu, Kushiro, Shibetsu, and Yufutsu mires in Hokkaido. We sampled leaves to analyze ploidy with a flow cytometry. For chromosome observations in root tips, we harvested scions from plants obtained from HFRI and the Bekanbeushi, Kushiro, Senjogahara, and Yufutsu mires, and propagated individual plants by cutting.

DNA ploidy analysis and DNA content measurements using flow cytometry

DNA ploidy level was analyzed using 4′, 6-diamidino-2-phenylindole (DAPI) stained flow cytometry (Partec PA; Partec GmbH, Münster, Germany). According to the concept of Suda et al. (2006), we use the term "DNA ploidy level". The prefix 'DNA' indicates that ploidy levels are mostly inferred from nuclear DNA content without knowledge of the exact chromosome numbers. Fresh young leaf was chopped with a 0.2 ml drop of nuclei extraction buffer (CyStain UV precise P; Partec, Münster, Germany). After filtration through a 30-μm nylon mesh, crude nuclear samples were stained with 0.8 ml DAPI solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) PVP K-30, 0.1% (v/v) Triton X-100, and 2 mg l^{-1} DAPI (pH 7.5) (Mishiba et al. 2000). After incubation for five minutes at room temperature, DNA ploidy level was analyzed with a flow cytometry. A total of more than 5000 cells were analyzed. Leaves

of Yufutsu No. 47 ($2n = 4x = 36$) were used as the internal standard. Totally, 450 plants from 21 populations were examined. For each sample, two replicates were performed.

We also performed the measurement of relative DNA content by flow cytometry as described above, but with slight modifications. The volume of nuclei extraction buffer applied was increased up to 0.4 ml. Fresh leaves of *Capsicum annuum* L. (cv. 'Kyonami') were used as the internal standard. Totally, 14 diploid plants from 3 populations (the Bekanbeushi and Kushiro mires, and Betsukai) and 38 tetraploid plants from 9 populaions (Bihoro Pass, Mt. Daisetsu, Mt. Eboshidake, Mt. Yokotsudake, Taiki, and the Kiritappu, Senjogahara, Shibetsu, and Yufutsu mires) were investigated. Fresh young leaves were sampled at flowering season in May, 2009. The leaves of five individuals from Mt. Yokotsudake and Mt. Eboshidake were exceptionally sampled at fruiting season in August, 2009. A total of more than 5000 cells were analyzed in each measurement. Basically, when the coefficient of variation (CV) was within 5%, the analysis was accepted. Exceptionally, in measurement of some individuals from Mt. Yokotsudake, Mt. Eboshidake, Kushiro and Bekanbeushi mire, CV of more than 5% was accepted because of limited sample amount. For each sample, three replicates were performed within 1 day.

Relative DNA content was estimated by the ratio between the fluorescence intensity of the sample and internal standard: sample relative DNA content = sample peak mean / standard peak mean. The relative DNA content was then calculated for each sample as the average of the three replications.

Statistical tests were performed using the SPSS 16.0J program. In DNA diploid populations, differences of the relative DNA content was analyzed using non-parametric Mann-Whitney's U-test, with $P < 0.05$ as the level of statistical significance. The

differences in relative DNA content among DNA tetraploid populations were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test, with $P < 0.01$ as the level of statistical significance. The relationship between the relative DNA content and altitude was investigated by Pearson's correlation coefficient test. The altitude is shown in Table 1. When the altitude ranged widely, the mean values were used for analysis.

Chromosome analysis

For counting the chromosome number, actively growing root tips were utilized for chromosome observation. Root tips were pretreated with ice water for 24 h and fixed with acetic acid:ethanol (1:3) at 4°C overnight. The fixed root tips were treatment with an enzyme mixture of 2% Cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Japan) and 0.5% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan) (Shibata and Hizume 2002) in the citrate buffer (0.01 M citric acid and 0.01 M trisodium citrate dehydrate), pH 4.5, at 37°C for 20 min. After treatment with the enzyme mixture, the root tips were rinsed with distilled water. Then, the root tips were squashed with forceps in a drop of 45% acetic acid on slide glass, covered with cover glass, and squashed again. Cover glasses were removed by freezing glass slides in liquid nitrogen, slides were dried at 37°C. For staining, a drop of DAPI solution [0.233 g 1,4-diazabicyclo(2.2.2)-octane, 1 ml 0.2 M Tris-HCl, pH 8.0, 9 ml glycerol, 0.5 μg ml^{-1} of DAPI] (Sahara et al. 2003) was put on the slide glass, and covered with cover glass. Preparations were observed under a fluorescence microscope (Aixo imager M1, Carl Zeiss, Oberkochen, Germany). Chromosome number was counted for 1–2 representative individuals from 11 populations. For each sample, 5–10 measurements

were recorded.

Results

DNA ploidy level analysis by flow cytometry, and chromosome counts

We measured the DNA ploidy levels of 450 plants from 21 locations with flow cytometry, and we obtained two distinct groups with regard to the relative DNA content (Table 1). All plants harvested in the Bekanbeushi and Kushiro mires showed 2Cx DNA content (Table 1, Fig. 2). In this study, description of genome size is expressed as C_X-value, which is designated as DNA content of a monoploid genome with chromosome number *x*, according to Greilhuber et al. (2005). Of the plants from Kiritappu mire and Betsukai, two types of DNA ploidy levels were obtained. One strain from Kiritappu mire from the collection at Hokkaido University had 2Cx DNA content, whereas other plants from Kiritappu mire had 4Cx DNA content (Table 1). In addition, one strain from Betsukai (population code, BT-1) and one strain from Betsukai from the collection at HFRI (population code, BT-2) had 2Cx DNA content, whereas other Betsukai strains from HFRI had 4Cx DNA content (Table 1). Plants of all strains with 4Cx DNA content were found in Bihoro Pass; Mt. Daisetsu; Mt. Eboshidake; Mt. Piyashiri; Mt. Yokotsudake; Taiki; and the Yufutsu, Senjogahara, and Shibetu mires (Table 1). Flow cytometric analysis revealed that 65 plants had 2Cx DNA content, while 385 had 4Cx DNA content (Table 1).

The results of the flow cytometric analysis were confirmed by determining the chromosome number. The chromosome number was counted for representative individuals from 11 populations. This analysis confirmed that diploid ($2n = 2x = 18$) and tetraploid ($2n = 4x = 36$) populations existed in the natural populations of *L. caerulea*

(Table 1, Fig. 3). The populations from the Bekanbeushi and Kushiro mires and one strain from Kiritappu mire obtained from Hokkaido University were diploid (Table 1, Fig. 3a). Other strains from Kiritappu mire from Hokkaido University and HFRI were tetraploid (Table 1, Fig. 3b). In addition, all the plants obtained from other populations were tetraploid (Table 1).

DNA content variation

Next, we measured of relative DNA content in *L. caerulea* by using fresh leaves of *Capsicum annuum* (cv. 'Kyonami') as an internal standard (Fig. 4). The relative DNA content in DNA diploid and DNA tetraploid populations is shown in Table 2. The mean relative DNA content in 14 individuals from three DNA diploid populations (the Bekanbeushi and Kushiro mires and Betsukai) was 0.257. The mean relative DNA content in 38 individuals from 9 DNA tetraploid populations (Bihoro Pass, Mt. Daisetsu, Mt. Eboshidake, Mt. Yokotsudake, Taiki, and the Kiritappu, Senjogahara, Shibetsu, and Yufutsu mires) was 0.528. The variation of relative DNA content within same DNA ploidy level was larger in DNA tetraploid populations than that of DNA diploid populations. The coefficient of variation of relative DNA content was 0.88 and 4.55 in DNA diploid and DNA tetraploid populations, respectively. The relative DNA content within DNA diploid populations varied 1.024-fold (from 0.254 to 0.260) at maximum, while those of DNA tetraploid populations varied 1.157-fold (from 0.492 to 0.569).

The relative DNA content of individual DNA tetraploid populations is shown in Table 3 and Figure 5. The relative DNA content within DNA tetraploid populations varied 1.157-fold at maximum (Table 2), however, only slight variation (1.002 to 1.052-fold) was observed within a single population (Table 3). The coefficient of

variation of relative DNA content was 0.2–1.8 within a single population (Table 3). The differences in relative DNA content among five DNA tetraploid populations (Mt. Daisetsu, Mt, Yokotsudake, and the Kiritappu, Shibetsu, and Yufutsu mires) were analyzed using one-way ANOVA followed by Bonferroni's test. The populations from Bihoro Pass, Mt. Eboshidake, Senjogahara mire, and Taiki were excluded from the statistical analysis because of low sample number. The Bonferroni test ($P < 0.01$) showed significant differences among DNA tetraploid populations (Table 3, Fig. 5). DNA tetraploid populations could divide into three groups; 1) Mt. Daisetsu, 2) Mt. Yokotsudake and Yufutsu mire, 3) Kiritappu mire and Shibetsu mire. In DNA diploid populations, no significant differences between Bekanbeushi mire and Kushiro mire were found from the statistical analysis by Mann-Whitney's U-test. Population from Betsukai was excluded from the statistical analysis because of low sample number.

The relationship between the relative DNA content of the DNA tetraploid populations and altitude was determined with in the 38 individuals from 9 DNA tetraploid populations. The relative DNA content in the DNA tetraploids was significantly correlated with altitude ($r = -0.684$, $P < 0.001$, n = 38), indicating that DNA contents were smaller as altitude increases (Fig. 6).

Discussion

Distribution of diploid and tetraploid plants

In the present study, ploidy study revealed that diploid ($2n = 2x = 18$) and tetraploid ($2n = 4x = 36$) plants of *L. caerulea* were distributed in Japan. Worldwide, the diploid and tetraplaoid of *Lonicera* subsection *Caeruleae* were found in Eurasia to North America (Plekhanova et al. 1992; Solovyeva and Plehanova 2003). So far, diploid species were

found in only four locations, such as Central Asian, Transbaikalian, South-Ussurian (Plekhanova 2000; Plekhanova et al. 1992), and Alberta, Canada (Solovyeva and Plehanova 2003). In addition to previous reports, our study found out distribution of diploid *L. caerulea* in Hokkaido, Japan.

In previous reports (Plekhanova 2000; Plekhanova et al. 1992), dominant tetraploid populations of *Lonicera* subsection *Caeruleae* were found in Eurasia. It has also been shown that tetraploid plants spread to more northern and alpine habitats than diploid plants do. Diploid plants occurred on territories unaffected by glaciers, and are located in proximity to Central China, the center of origin of bushy *Lonicera* (Plekhanova 2000; Plekhanova et al. 1992). In the case of Japan, DNA tetraploid plants were distributed in wide area of Hokkaido, and mainland of Japan, while DNA diploid plants occurred only in Betsukai, Bekanbeushi mire, Kiritappu mire and Kushiro mire located in eastern Hokkaido. The habitats of DNA tetraploid plants were lowland mires, around lowland mires, developed mires, and mountain regions (mountain mire or rocky area) (Table 1). On the other hand, DNA diploid plants were found only in lowland mire (Table 1). In many plant species, the geographical distributions of diploid and tetraploid plants differ (Lewis 1980). In the present study, the populations in the mountainous habitats (Bihoro Pass, Mt. Daisetsu, Mt. Eboshidake, Mt. Piyashiri, Mt. Yokotsudake, and Senjogahara mire) were only tetraploid, suggesting that these populations prefer in mountainous regions. In addition, the wide area of distribution in various environments, similar to that shown in previous studies in Eurasia (Plekhanova 2000; Plekhanova et al. 1992), suggested the adaptability of the tetraploid plants.

Natural triploid populations of *Lonicera* subsection *Caeruleae* have not been reported. Similarly, no triploid plant was observed in the present study. In general,

triploid plants are produced by the crossing between diploid and tetraploid or by the union of unreduced (2*n*) and reduced (*n*) gametes in diploid plants (reviewed by Ramsey and Schemske 1998). However, crosses between diploid and tetraploid plants are often unsuccessful because of abnormal seed development (reviewed by Haig and Westoby 1991; reviewed by Ramsey and Schemske 1998). To investigate the possibility of triploid induction, we attempted to cross between diploid and tetraploid *L. caerulea* plants. The number of mature seeds obtained from the reciprocal cross was substantially less than that of 2*x*-2*x* and 4*x*-4*x* plants (unpublished data). Low fruit and seed setting as well as the low viability of seedlings from crosses between diploid and tetraploid individuals of *Lonicera* subsection *Caeruleae* were also reported by Plekhanova (2000). The lack of a triploid population may be because of the low success of seed production and reduced survival of triploid seedlings.

DNA content variations among DNA tetraploid populations

In the present study, variations in the relative DNA content were detected by flow cytometry among the DNA tetraploid populations (Table 3, Fig. 5). Genomic size variation was first mentioned by Evans et al. (1966). It has been reported at the interspecific and intraspecific levels (Knight et al. 2005). Genomic size variations within the same species have been reported in some species such as those belonging to Poaceae (Ceccarelli et al. 1992; Creber et al. 1994; Reeves et al. 1998; Sugiyama and Yamada 2003; Šmarda and Bureš 2006) and Asteraceae (Suda et al. 2007). These studies have shown the correlation of genomic size with geographical condition, climate, or vegetation. The correlation between genomic size and altitude was reported in tetraploid populations of *Dactylis glomerata* L. (Creber et al. 1994; Reeves et al. 1998),

i.e., the genomic size decreases with increasing altitude. Similarly, the relative DNA contents in the DNA tetraploids were smaller as altitude increases in *L*. *caerulea* (Fig. 6). Murray (2005) stated that varying DNA content could be correlated with eco-geographic variations, suggesting that the varying DNA content is adaptive and may be an example of incipient speciation. The genome downsizing following polyploid formation may be a widespread phenomenon (Leitch and Bennett 2004). The changes of DNA content could be caused by activation of transposons, homoeologous recombination, or elimination of specific DNA sequences (Leitch and Bennett 2004). Solovyeva and Plehanova (2003) examined the karyotype of tetraploid *Lonicera* subsection *Caeruleae* plants and observed variations in chromosome size, number of satellites, and telomeres, which might confirm our results regarding the DNA content variability in DNA tetraploids.

However, DNA content variation could be explain by other factors, such as ancient introgression with related species (Suda et al., 2007), or dissimilar levels of secondary metabolites that may have interfered with DNA fluorochromes (Kubátová et al., 2008). Aneuploidy is also one of the possible explanation about DNA content variation, although no aneuploidy was observed in the present study as well as in previous reports of *Lonicera* subsection *Caeruleae*. Further studies including phylogenetic analysis and analyses of biochemical compounds affecting DNA staining are required for reliable conclusions about relative DNA content variation in *L*. *caerulea*.

Acknowledgements

The authors thank Drs. K. Sahara and F. Shibata for their valuable suggestions

on chromosome observation. We also thank to Drs. T. Sato and Y. Wakita (Hokkaido Forestry Research Institute) and “Nikko Botanical Garden”, Graduate School of Science, the University of Tokyo, and Shibetsu Town for providing plant materials of *Lonicera caerulea*. We are very grateful to A. Takai (Kiritappu Wetland Center) and T. Shibuya (Akkeshi Waterfowl Observation Center) for their helps in collecting the plant materials. We gratefully acknowledge Prof. H. Nakashima, H. Hori, H. Tamura, M. Ikuta, H. Nakano, S. Takamushi and T. Takahashi for the technical assistances on the University farms. This work was supported in part by grants from Takeda Scientific foundation, Grant-in-Aid for Scientific Research of Lake Akkeshi and Bekanbeushi Wetland, a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports Science and Technology (MEXT), and Global COE Program “Establishment of Center for Integrated Field Environmental Science”, MEXT, Japan.

References

Ammal EKJ, Saunders B (1952) Chromosome numbers in species of *Lonicera*. Kew Bulletin 7:539–541

Anetai M, Ogawa H, Hayashi T, Aoyagi M, Chida M, Muraki M, Yasuda C, Yabunaka T, Akino S, Yano S (1996) Studies on wild plants traditionally used by the Ainu people (Part Ⅰ): Contents of vitamins A, C and E in edible plants. Report of the Hokkaido Institute of Public Health 46:34–39 (in Japanese with English summary)

Ceccarelli M, Falistocco E, Cionini PG (1992) Variation of genome size and organization within hexaploid *Festuca arundinacea*. Theor Appl Genet 83:273–278

Chaovanalikit A, Thompson MM, Wrolstad RE (2004) Characterization and quantification of anthocyanins and polyphenolics in blue honeysuckle (*Lonicera*

caerulea L.). J Agr Food Chem 52:848–852

Creber HMC, Davies MS, Francis D, Walker HD (1994) Variation in DNA C value in natural populations of *Dactylis glomerata* L. New Phytol 128:555–561

Evans GM, Durrant A, Rees H (1966) Associated nuclear changes in the induction of flax genotrophs. Nature 212:697–699

Greilhuber J, Doležel J, Lysák MA, Bennett, MD (2005) The origin, evolution and proposed stabilization of terms 'Genome size' and 'C-value' to describe nuclear DNA contents. Ann Bot 95:255–260

Haig D, Westoby M (1991) Genomic imprinting in endosperm: its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixes. Philos Trans R Soc London B 333:1–13

Hara H (1983) Section 3. Caeruleae. In: Ginkgoana, Contributions to the flora of Asia and the pacific region No. 5, A revision of Caprifoliaceae of Japan with reference to allied plants in other districts and the Adoxaceae, Academia Scientific Book Inc., Tokyo, Japan, pp 79–84

Knight CA, Molinari NA, Petrov DA (2005) The large genome constraint hypothesis: Evolution, ecology and phenotype. Ann Bot 95:177–190

Kubátová B, Trávníček P, Bastlová D, Čurn V, Jarolímová V, Suda J (2008) DNA ploidy-level variation in native and invasive populations of *Lythrum salicaria* at a large geographical scale. J Biogeogr 35:167-176

Leitch IJ, Bennett MD (2004) Genome downsizing in polyploid plants. Biol J Linn Soc 82:651-663

Lewis WH (1980) Polyploidy in species populations. In: W. H. Lewis [ed.], Polyploidy:

Biological Relevance, Plenum Press, New York, USA, pp 103–147

Mishiba K, Ando T, Mii M, Watanabe H, Kokubun H, Hashimoto G, Marchesi E (2000) Nuclear DNA content as an index character discriminating taxa in the genus *Petunia sensu* Jussieu (Solanaceae). Ann Bot 85:665–673

Murray BG. (2005) When does intraspecific C-value variation become taxonomically significant? Ann Bot 95:119–125

Naugžemys D, Žilinskaitė S, Denkovskij J, Patamsytė J, Literskis J, Žvingila D (2007) RAPD based study of genetic variation and relationships among *Lonicera* germplasm accessions. Biologija 53:34–39

Plekhanova MN (2000) Blue honeysuckle (*Lonicera caerulea* L.) – A new commercial berry crop for temperate climate: genetic resources and breeding. Acta Hortic 538:159-164

Plekhanova MN, Solovyeva LV, Mochalova OV (1992) Chromosome numbers and distribution area of *Lonicera* subsect. *Caeruleae* (Caprifoliaceae). Botanicheskii Zhurnal 77:1–11 (in Russian with English summary)

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst 29:467-501

Ramsey J, Schemske DW (2002) Neopolyploidy in flowering plants. Annu Rev Ecol Syst 33:589-639

Reeves G., Francis D, Davies MS, Rogers HJ, Hodkinson TR (1998) Genome size is negatively correlated with altitude in Natural populations of *Dactylis glomerata*. Ann Bot 82 (Supplement A):99–105

Sahara K, Yoshido A, Kawamura N, Ohnuma A, Abe H, Mita K, Oshiki T, Shimada T, Asano S, Bando H, Yasukochi Y (2003) W-derived BAC probes as a new tool for

identification of the W chromosome and its aberrations in *Bombyx mori*. Chromosoma 112:48–55

Sato T (1985) Hokkaido ni okeru Kurominouguisukagura · Keyonomi ni tuite (*Lonicera caerulea* var. *emphyllocalyx* and *L. caerulea* var. *edulis* in Hokkaido.) Wild Life Rep 2:47–51 (in Japanese)

Shibata F, Hizume M (2002) The identification and analysis of the sequences that allow the detection of *Allium cepa* chromosomes by GISH in the allodiploid *A. wakegi*. Chromosoma 111:184–191

Šmarda P, Bureš P (2006) Intraspecific DNA content variability in *Festuca pallens* on different geographical scales and ploidy levels. Ann Bot 98:665–678

Solovyeva LV, Plehanova MN (2003) Investigation of karyotype of the blue honeysuckle (*Lonicera* subsect. *caeruleae*, Caprifoliaceae). Cytol Genet 37:30–38

Suda J, Krahulcová A, Trávníček P, Krahulec F (2006) Ploidy level versus DNA ploidy level: an appeal for consistent terminology. Taxon 55:447-450

Suda J, Weiss-Schneeweiss H, Tribsch A, Schneeweiss GM, Trávníček P, Schönswetter P (2007) Complex distribution patterns of di-, tetra-, and hexaploid cytotypes in the European high mountain plant *Senecio carniolicus* (Asteraceae). Am J Bot 94:1391–1401

Sugiyama S, Yamada T (2003) Variation in nuclear DNA content in natural populations of orchardgrass (*Dactylis glomerata* L.) in the eastern part of Hokkaido, Japan. Grassland Sci 49:129–133

Terahara N, Sakanashi T, Tsukui A (1993) Anthocyanins from the berries of Haskaap, *Lonicera caerulea* L. J Home Econ Jpn 44:197–201

Thompson MM, Chaovanalikit A (2003) Preliminary observations on adaptation and

nutraceutical values of blue honeysuckle (*Lonicera caerulea*) in Oregon, USA. Acta Hortic 626:65–72

Table 1. Geographical locations and ploidy levels of *Lonicera caerulea* in Japan.

Population codes	Populations	Habitat	Geographical coordinates Latitude (°N)	Longitude (°E)	Altitude (m)	n	DNA Ploidy level 2*x*	4*x*	Chromosome number (2*n*)	Voucher number
Mainland										
SE[a]	Senjogahara mire	Area surrounding Senjogahara mire	36.46	139.26	1390–1400	3	0	3	36	SE1-SE3
Hokkaido										
BK–1	Bekanbeushi mire	Alder forest	43.10	144.51	0–10	4	4	0	18	BET1-BET4
BK–2	Bekanbeushi mire	Alder forest	43.06	144.55	0–10	5	5	0	—	BEI1-BEI5
BK–3	Bekanbeushi mire	Developed area along R44	43.06	144.58	0–10	23	23	0	—	BEI6-BEI28
BT–1	Betsukai	Area drained by agricultural development in Fuhren mire	43.16	145.11	20	1	1	0	—	KA1
BT–2[b]	Betsukai	—	—	—	—	3	1	2	—	BEK1-BEK3
BP[b]	Bihoro Pass	Rocky area	43.38	144.15	500–525	1	0	1	36	BI1
KI–1	Kiritappu mire	High moor near estuary of Biwase River	43.03	145.04	0–2.5	29	0	29	—	KIB1-KIB29
KI–2	Kiritappu mire	Intermediate moor in Sakaki, Hamanaka	43.07	145.06	0–2.5	47	0	47	—	KIS1-KIS47
KI–3[b]	Kiritappu mire	—	43.02–43.08	145.01–145.06	0–10	3	0	3	36	K1-K3
KI–4[c]	Kiritappu mire	—	43.02–43.08	145.01–145.06	0–10	2	1	1	18, 36	K4-K5
KU	Kushiro mire	High moor, Alder forest adjacent to high moor	43.06	144.20	5–10	30	30	0	18	KU1-KU30
MD[b]	Mt. Daisetsu	Around mountainous mire	43.36–43.48	142.53	1700–1800	6	0	6	36	TS1-TS6
ME	Mt. Eboshidake	Rocky area around mountaintop	41.55	140.47	1070-1078	3	0	3	—	EB1-EB3
MP[b]	Mt. Piyashiri	Rocky area	44.24–44.26	142.33–142.36	600–987	1	0	1	—	PI1
MY–1	Mt. Yokotsudake	Rocky area around mountaintop	41.56	140.46	1120–1150	7	0	7	—	YO1-YO7
MY–2[b]	Mt. Yokotsudake	Rocky area around mountaintop	41.56	140.46	1120–1150	3	0	3	36	YO8-YO10
SH	Shibetsu mire	High moor in Pho River Historic & Nature Park	43.41	145.06	0–5	64	0	64	—	SI1-SI64
TA[b]	Taiki	Alder forest, *Quercus crispula* and *Q. dentata* forest	42.31–42.33	143.28–143.30	0–70	4	0	4	36	TK1-TK4
YU–1	Yufutsu mire	Area drained by industrial development	42.40	141.45	5	200	0	200	36	TO1-TO200
YU–2[b]	Yufutsu mire	—	—	—	—	11	0	11	36	Y1-Y11

[a] The plants were provided by Nikko Botanical Garden (NBG). The habitat, geographical coordinates, and altitude were determined from

sample recordings from NBG.

[b] All the plants were provided by the Hokkaido Forestry Research Institute (HFRI). These plants were collected by T. Sato. The habitat, geographical coordinates, and altitude were determined from Sato's report (1985).

[c] All the plants were obtained from collections at Hokkaido University. These plants were collected by T. Sato. The geographical coordinates and altitude were determined from Sato's report (1985).

Dashes indicate no data.

Table 2. Relative DNA content in DNA diploid and DNA tetraploid populations estimated by flow cytometry.

Ploidy level	Number of population	n	Mean ± SD	Minimum	Maximum	Difference within population (fold)[a]	Coefficient of variation[b]
DNA diploid	3	14	0.257 ± 0.002	0.254	0.260	1.024	0.88
DNA tetraploid	9	38	0.528 ± 0.024	0.492	0.569	1.157	4.55

Relative DNA content was estimated by the ratio between the fluorescence intensity of a sample and internal standard: sample relative DNA content = sample peak mean / standard peak mean.

[a] Differences of relative DNA content (fold) within population was calculated as Maximum relative DNA content / Minimum relative DNA content.

[b] Coefficient of variation of relative DNA content was calculated as (SD / Mean) × 100.

Table 3. Relative DNA content in DNA tetraploid populations as estimated by flow cytometry.

Populations	n	Mean ± SD		Minimum	Maximum	Difference within population (fold)[c]	Coefficient of variation[b]
Senjogahara mire	1	0.511	–	—	—	—	—
Bihoro Pass	1	0.496	–	—	—	—	—
Kiritappu mire	6	0.564 ± 0.005	c	0.557	0.569	1.022	0.89
Mt. Daisetsu	5	0.497 ± 0.004	a	0.492	0.501	1.018	0.80
Mt. Eboshidake	2	0.510 ± 0.001	–	0.510	0.511	1.002	0.20
Mt. Yokotsudake	6	0.518 ± 0.007	b	0.513	0.531	1.035	1.35
Shibetsu mire	6	0.557 ± 0.010	c	0.537	0.565	1.052	1.80
Taiki	1	0.535	–	—	—	—	—
Yufutsu mire	10	0.518 ± 0.004	b	0.513	0.529	1.031	0.77

Relative DNA content was estimated by the ratio between the fluorescence intensity of a sample and internal standard: sample relative DNA content = sample peak mean / standard peak mean.

[a] Differences of relative DNA content (fold) within population was calculated as Maximum relative DNA content / Minimum relative DNA content.

[b] Coefficient of variation of relative DNA content was calculated as (SD / Mean) × 100.

Means ± SD followed by the same letter are not significantly different (Bonferroni's test, $P < 0.01$).

Dashes indicate no data.

Figure Legends

Fig. 1. Map of Japan showing sampling locations of *Lonicera caerulea* described in Table 1. The Circles indicate sampling locations. The white and black circles show the diploid and tetraploid populations, respectively. The letters indicate the population codes described in Table 1. The squares indicate other habitats of *L. caerulea* described in previously reports (Hara 1983; Sato 1985).

Fig. 2. Histograms of the relative fluorescence intensity of nuclei isolated from the leaves of *L. caerulea*. Peak of 2Cx and 4Cx were from Kushiro mire strain and internal standard, respectively. The leaves of Yufutsu No. 47 ($2n = 4x = 36$) were used as the internal standard.

Fig. 3. Metaphase chromosomes of *L. caerulea* plants obtained from individual locations. (a) Kushiro mire ($2n = 2x = 18$). (b) Kiritappu mire ($2n = 4x = 36$). Bars = 5 μm.

Fig. 4. Histograms of the relative fluorescence intensity of nuclei isolated from the leaves of *L. caerulea* and internal standard. (a) DNA diploid plants from Kushiro mire (b) DNA tetraploid plants from Shibetsu mire. The peak of *L. caerulea* is marked with an asterisk. Fresh leaves of *Capsicum annuum* (cv. 'Kyonami') were used as internal standard.

Fig. 5. Box plot graph of relative DNA content of DNA tetraploid plants from different populations. Thick line across the box indicates the median, the box contains the values

between the 25% to 75%, and the whiskers extend to the highest and lowest values, excluding the outliers. The asterisk represents the outlier. The same letters above the box-plots indicate that there is no significant differences (Bonferroni's test, $P < 0.01$). Dashes indicate no data.

Fig. 6. Relationship of relative DNA contents with altitude. The samples were 38 individuals from nine DNA tetraploid populations described in Table 3. The relative DNA contents were negatively correlated with altitude ($r = -0.684$, $P < 0.001$, n = 38).

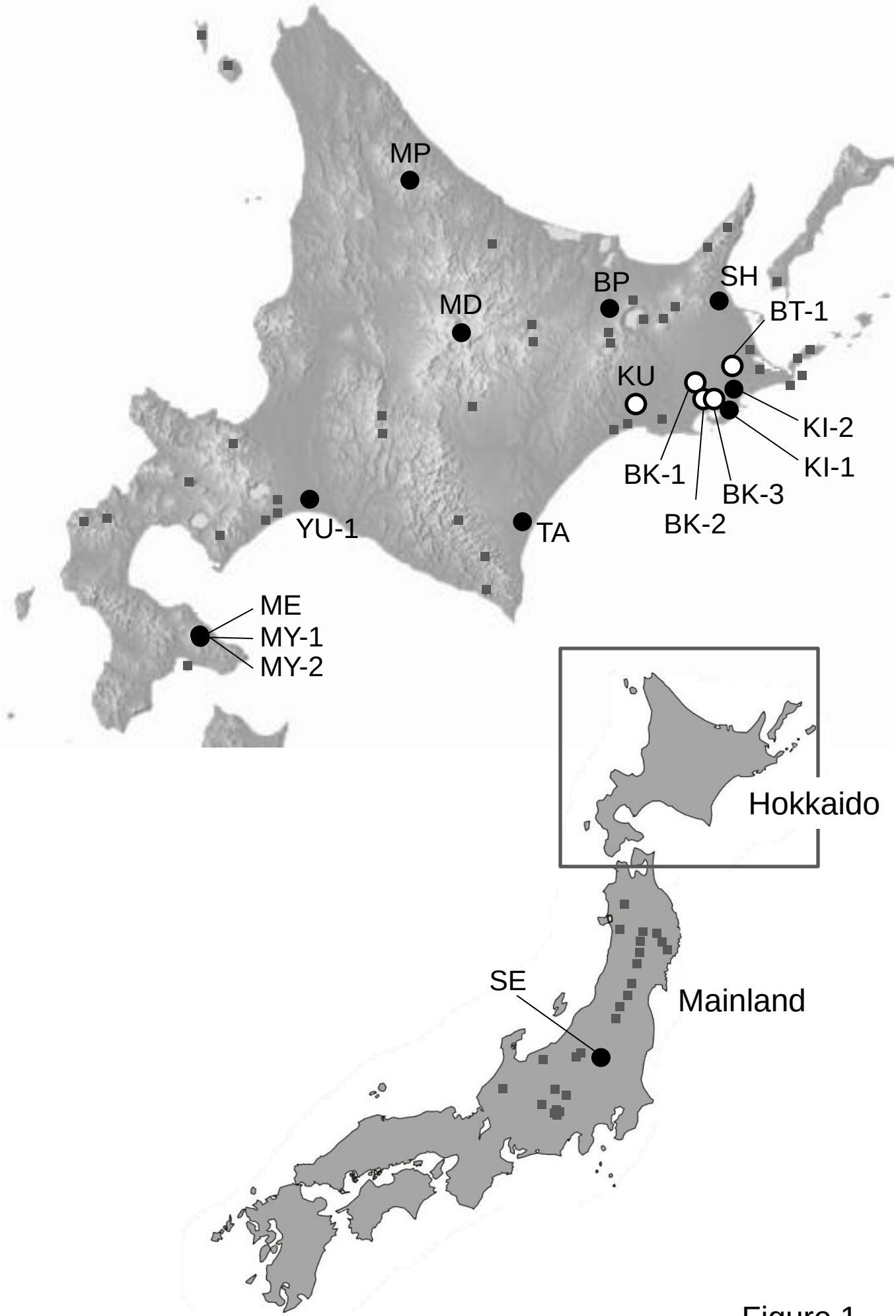
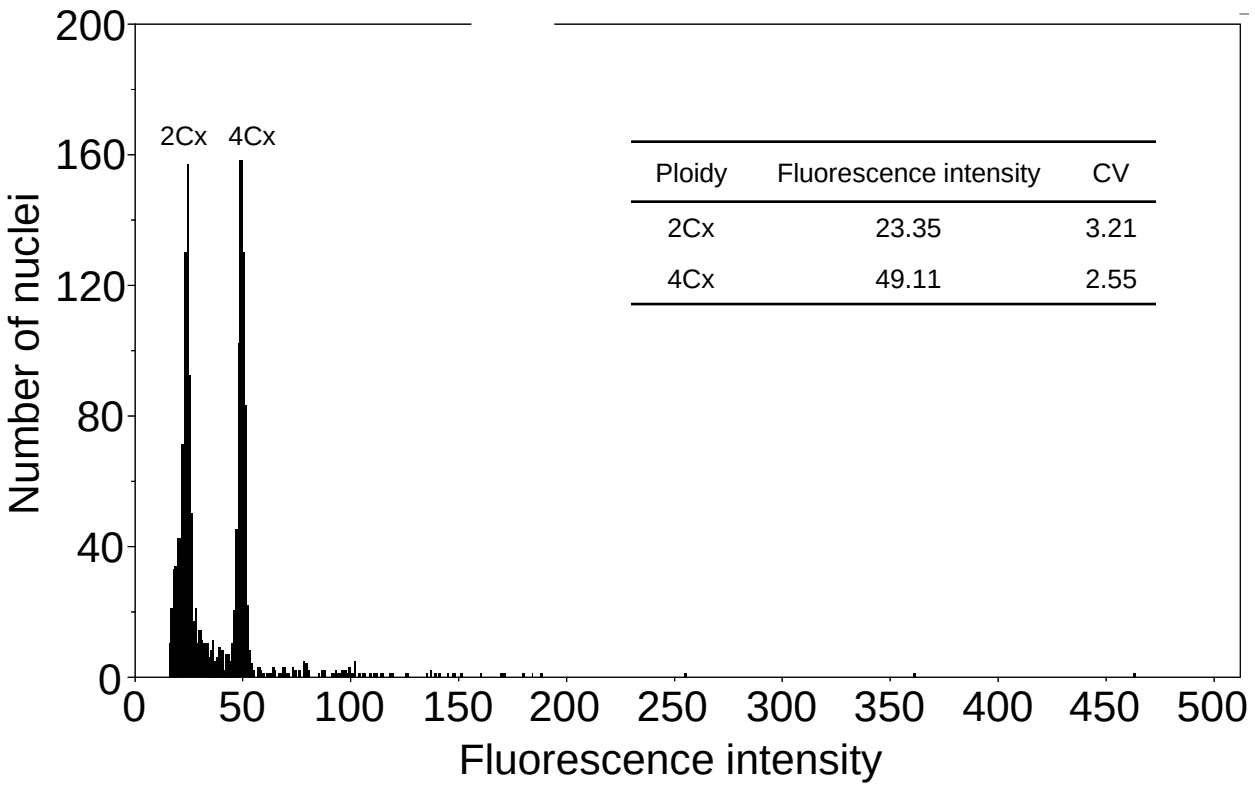

MP
MD
BP
SH
BT-1
KU
KI-2
KI-1
BK-1
BK-3
BK-2
YU-1
TA
ME
MY-1
MY-2
Hokkaido
SE
Mainland

Figure 1



Ploidy	Fluorescence intensity	CV
2Cx	23.35	3.21
4Cx	49.11	2.55

Figure 2

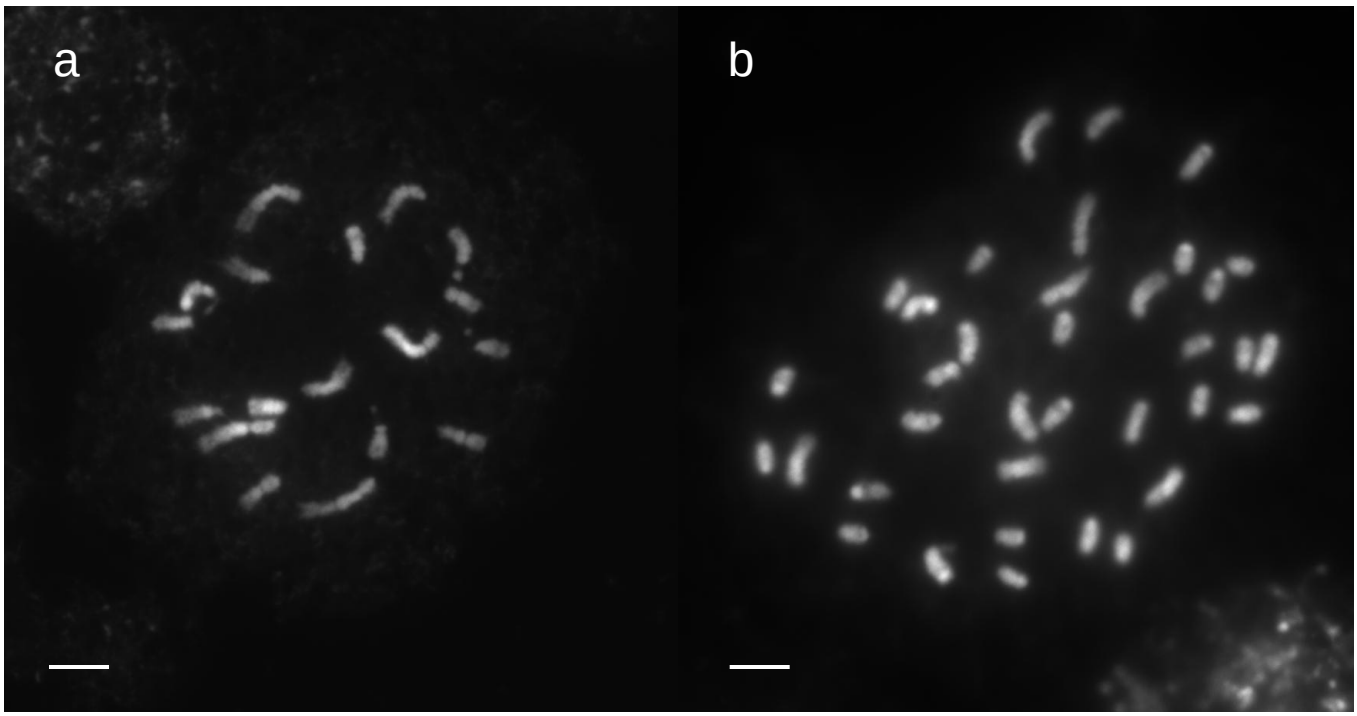
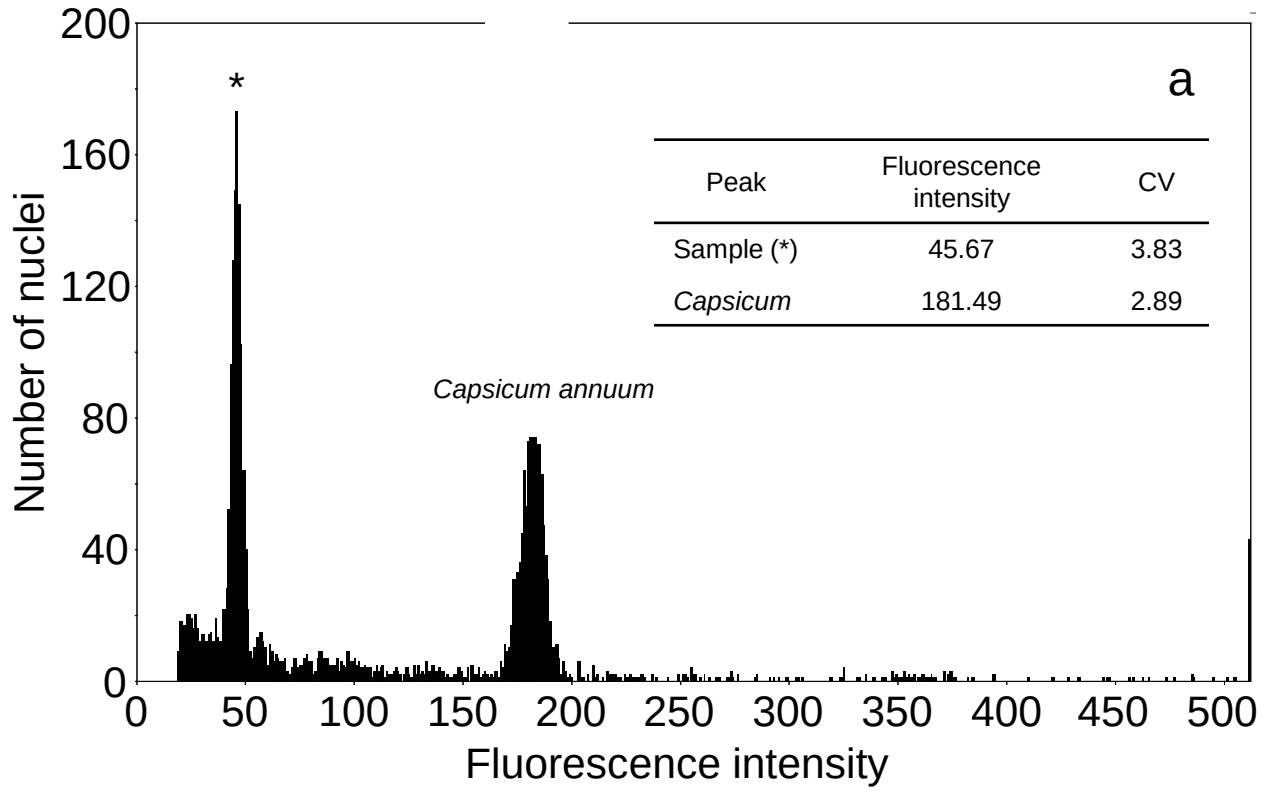
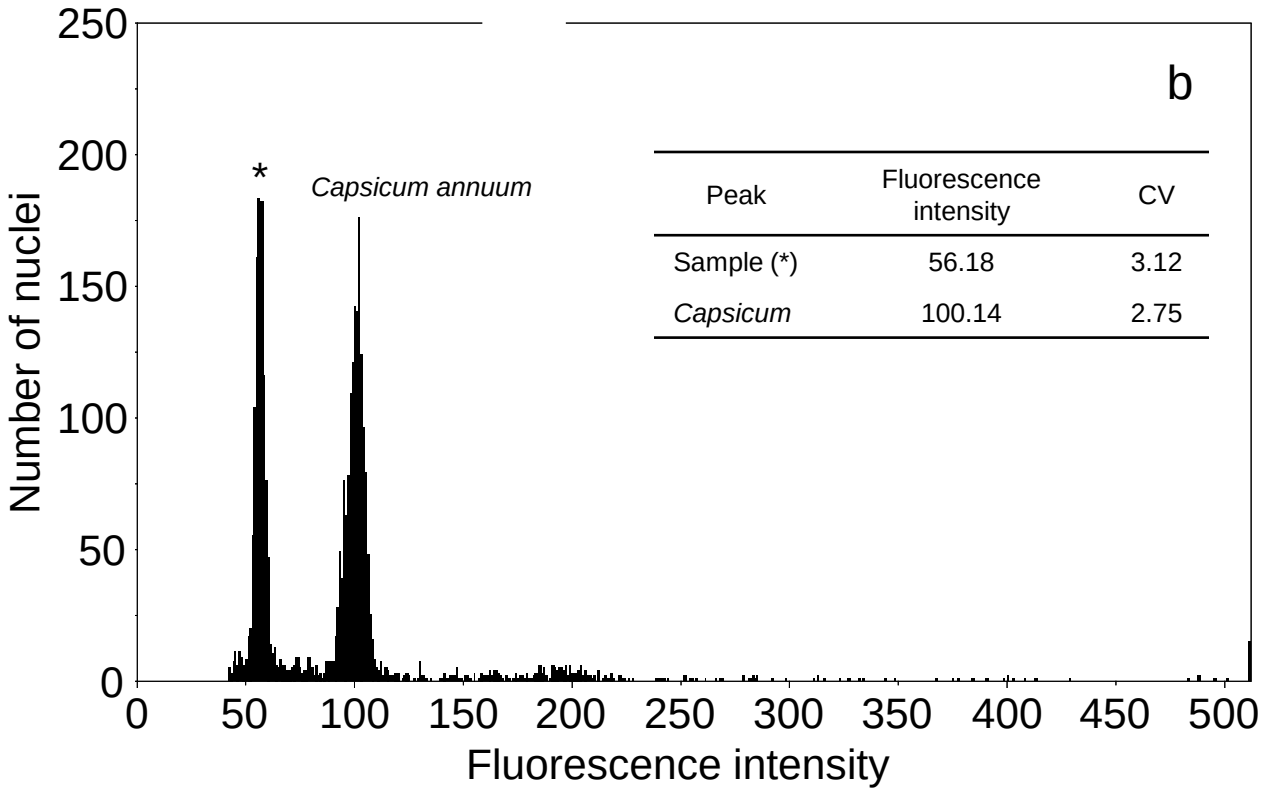


Figure 3



Peak	Fluorescence intensity	CV
Sample (*)	45.67	3.83
Capsicum	181.49	2.89



Peak	Fluorescence intensity	CV
Sample (*)	56.18	3.12
Capsicum	100.14	2.75

Figure 4

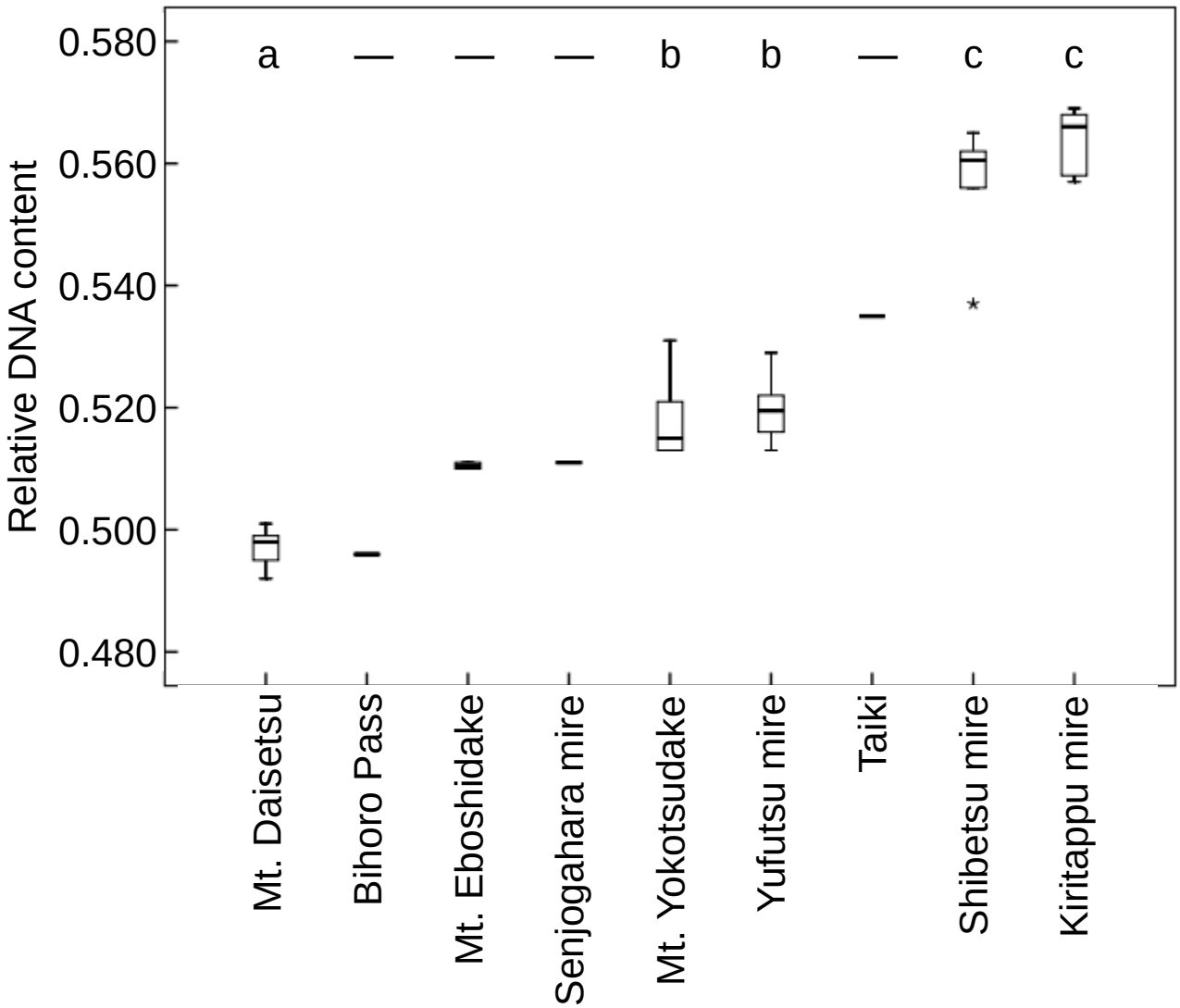

Relative DNA content
0.580
0.560
0.540
0.520
0.500
0.480
a
b
b
c
c
Mt. Daisetsu
Bihoro Pass
Mt. Eboshidake
Senjogahara mire
Mt. Yokotsudake
Yufutsu mire
Taiki
Shibetsu mire
Kiritappu mire

Figure 5

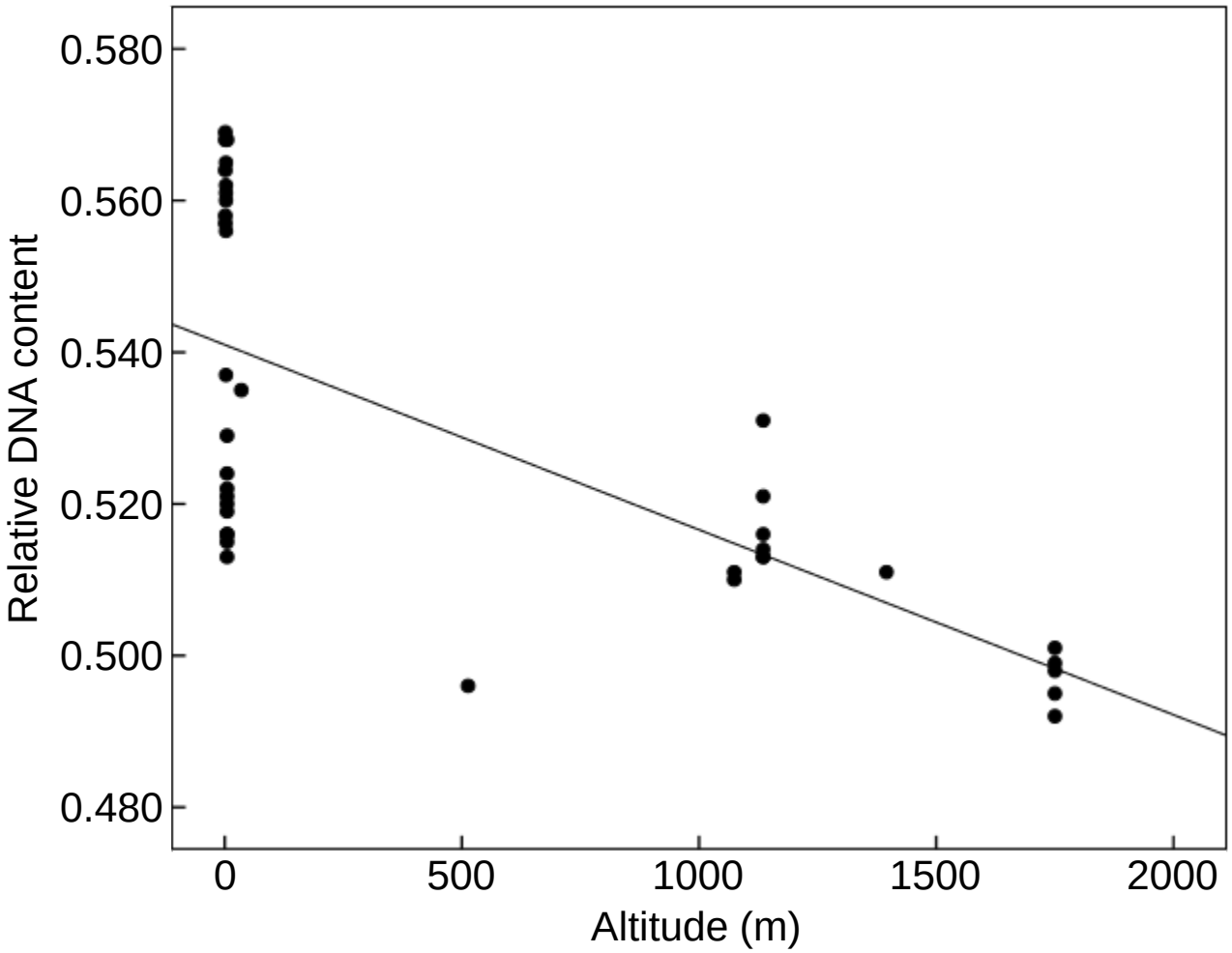

0.580
0.560
0.540
0.520
0.500
0.480
Relative DNA content
0
500
1000
1500
2000
Altitude (m)

Figure 6