白山におけるアキノキリンソウとミヤマアキノキリンソウのリボゾーム遺伝子座とRAPD分析

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Miki Nakamura*, Junko Miyamoto**, Koji Murai***, Masumi Yamagishi*** and Kiichi Fukui**** : The rDNA Locus and the RAPDs Analysis of *Solidago virgaurea* ssp. *asiatica* and ssp. *leiocarpa* (Compositae) in Mt. Hakusan

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アキノキリンソウとミヤマアキノキリンソウのリボゾーム遺伝子座と RAPD 分析

Abstract

Solidago virgaurea L. (Compositae) has been divided into three subspecies on the bases of morphological variation associated with elevation in Japan. We collected two of them, *S. virgaurea* ssp. *asiatica* Kitamura and *S. virgaurea* ssp. *leiocarpa* (Benth.) Hultén, from their natural habitats on Mt. Hakusan, Japan. Chromosomal and genetic polymorphism were analysed by a fluorescence *in situ* hybridization (FISH) method and patterns of randomly amplified polymorphic DNAs (RAPDs). Karyotypes of both subspecies were very similar to each other and a 45 S ribosomal DNA (rDNA) locus was detected on a satellite chromosome. A phenogram generated by an unweighted pair-group method with arithmetrical averages (UPGMA) based on 67 polymorphic bands generated by the RAPD analysis did not discriminate the two subspecies. The two subspecies, *Solidago virgaurea* ssp. *asiatica* and ssp. *leiocarpa* could not be distinguished by the molecular methods currently employed.

Key words: Fluorescence *in situ* hybridization (FISH), 45 S rDNA-Random amplified polymorphic DNAs (RAPD), *Solidago virgaurea* ssp. *asiatica*, *Solidago virgaurea* ssp. *leiocarpa*.

Solidago virgaurea L. (Compositae) grows in Europe and Asia. It shows tremendous morphological variation throughout its distributional range. *Solidago virgaurea* L. ssp. *asiatica* Kitamura is distributed in China, Korea, and Japan, and *S. virgaurea* L. ssp. *leiocarpa* (Benth.) Hultén grows in China, Korea, Japan, Formosa, Krills, and Sahkalin. Systematic study on Japanese taxa has been based mainly on gross morphological features, such as the shape of the leaf and the involucral scale (Kitamura 1937, 1956; Hara 1952). Intraspecific variation of *S. virgaurea* in Japan has been studied, and three subspecies, three varieties, and one form were descrived: *S. virgaurea* ssp. *leiocalpa* Hultén, *S. virgaurea* f. *paludosa* (Honda) Kitamura, *S. virgaurea* var. *preaflorens* Nakai, *S. virgaurea* var. *coreana* (Nakai) Kitamura, *S. virgaurea* ssp. *gigantea* (Nakai) Kitamura, *S. virgaurea* ssp. *asiatica* Kitamura, and *S. virgaurea* var. *insularis* Kitamura (Nakai 1917, 1928; Hultén 1937; Kitamura 1937, 1956; Hara 1952). They are all diploids with a chromosome number of 2 n=18. (Hujiwara 1962; Kapoor and Beaudry 1966; Nishikawa 1979, 1988; Abe and Takasu 1983).

In central Japan, the ratio of dry weight of shoot to rhizome in *S. virgaurea* decreases with increasing elevation. There appear to be two ecological types of *S. virgaurea*, a lowland-subalpine type and an alpine type (Natori 1964).

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Solidago virgaurea ssp. *asiatica* and *S. virgaurea* ssp. *leiocalpa* were distinguished from each other based on gross morphology. *Solidago virgaurea* ssp. *asiatica* is usually distributed from the sea level to the subalpine zone, and *S. virgaurea* ssp. *leiocalpa* grows at the alpine zone. Plants having intermediate characters between the two subspecies are found in an intermediate zone between the alpine zone and the subalpine zone. The altitude of the intermediate zone was 1,600–2,500 m above the sea level in mountainous areas of central Japan (Takasu 1975; Takasu *et al.* 1980, 1982; Hayashi 1976, 1977, 1978; Suzuki and Teranuma 1986).

We report cytological and genetic polymorphism of two subspecies of *S. virgaurea*, *S. virgaurea* ssp. *asiatica* and *S. virgaurea* ssp. *leiocalpa*, by the karyotype analysis, fluorescence *in situ* hybridization (FISH), and randomly amplified polymorphic DNAs (RAPD) analysis. The aim of the study is to clalify whether the genetic border is found between these subspecies by cytological and PCR-based genetic analyses.

Materials and Methods

Plants of *S. virgaurea* were collected at Mt. Hakusan (Ishikawa prefecture, Japan). The sampling localities, their abbreviations, the altitude of the localities, and the number of samples are shown (Table 1). Twenty-four plants were collected from eight localities in their natural habitats along the trail on the southwestern slope of Mt. Hakusan. The altitude of the Oh-Nanji, which is located near the highest peak of Mt. Hakusan, was 2,670 m above sea level. The lowest was at Betto-Deai at an altitude of 1,260 m. The samples collected from Oh-Nanji (ON), Mizu-Yajiri (MY), and Midaga-Hara (MH) were identified as *S. virgaurea* ssp. *leiocarpa*, and those from Nakahanba-Rin-Do (RD) and Betto-Deai (BD) as *S. virgaurea* ssp. *asiatica* based on the gross morphology of the involucral scale (Kitamura 1937, 1956). The samples collected from Nanryuga-Baba (NB), Jin'nosuke-Goya (JG), and Betto-Nozoki (BN) were intermediate types between *S. virgaurea* ssp. *asiatica* and *S. virgaurea* ssp. *leiocarpa* based on gross morphology. In addition, *S. altissima* L. was collected in Nonoichi-machi, Ishikawa prefecture, and used as an outgroup or a control during RAPD analysis.

Root tips of *S. virgaurea* ssp. *asiatica* and *S. virgaurea* ssp. *leiocarpa* were fixed with the mixture of acetic acid and ethanol (1 : 3). They were macerated by the enzymatic mixture (2% Cellulase Onozuka RS, Yakult Honsya Co. Ltd., Japan, 1.5% Macerozyme R-200, Yakult Honsya Co. Ltd, and 0.3% Pectlyase Y-23, Seishin Pharmaceutical Ltd., Japan) and air dried. Chromosomes were stained with 2% Giemsa (Merch, Germany) /PBS (0.13 M NaCl, 0.07 M Na_2HPO_4, and 0.003 M NaH_2PO_4, pH 6.8) and used for the karyotype analysis. Accurate length of each chromosome of a cell was measured by a chromosome image analysing system, CHIAS (Fukui 1986, 1988)

Table 1. The names of the sampling localities, its abbreviations, the altitude of the localities, the number of samples

Localities	Abbreviation	Altitude (m)	No. of samples
Betto-Deai	BD	1260	3
Nakahanba-Rin-Do	RD	1520	3
Betto-Nozoki	BN	1810	3
Jin'nosuke-Goya	JG	1980	3
Nanryu-Baba	NB	2080	3
Midaga-Hara	MH	2350	3
Mizu-Yajiri	MY	2450	3
Oh-Nanji	ON	2670	3
			total 24

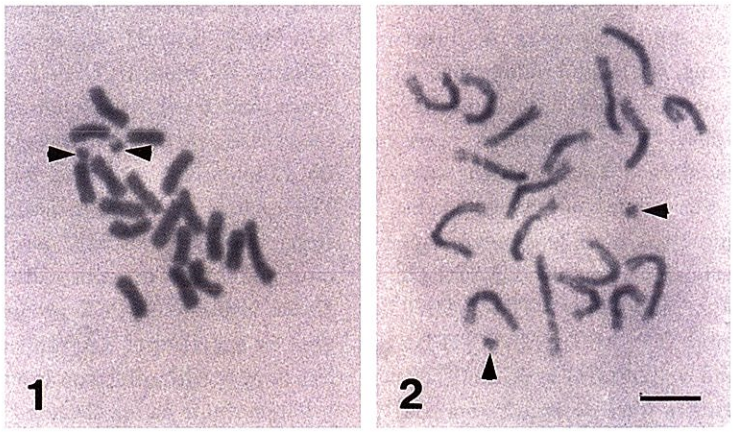
Then, 45 S rDNA loci were detected on the chromosomes in both subspecies using the direct cloning, direct labeling, FISH, and image analysing methods of Fukui *et al.* (1994 a, b), and Kamisugi *et al.* (1994). The procedures were slightly modified as follows: To amplify the 45 S rDNA probe, PCR was carried out under conditions using genomic DNA of *S. virgaurea* ssp. *asiatica* and two sets of primers (5'-CAATGGA TCCTCGTTAAGGG-3' and 5'-TACCTGGTTGAT CCTGCCAG-3'), and (5'-TAGTCATATGCTTG TCTCAAAGA-3' and 5'-TACCTGGTTGATCCTG CCAG-3'). The primers were designed from the consensus sequences of the barley 18 S and rice 17 S rDNA. The two cycling regimes each consisted of three steps: first, 94℃ for 1 min, 45℃ for 2 min, and 72℃ for 2 min, for 30 cycles, with a final step was 72℃ for 7 min, and second, 94℃ for 1 min, 37℃ for 2 min, and 72℃ for 2 min, were repeated for 30 cycles with a final step of 72℃ for 7 min. The probe was directly labeled with 70% substitution of biotin-11-dUTP (Enzo Diagnostics, USA). Hybridization mixture containing 100 ng of biotinylated 45 S rDNA probe in 50% formamide / 2 × SSC was dropped on a glass slide which was then placed on a thermal cycler and heated at 70℃ for 6 min and at 37℃ for 18 h. The detection of hybridization signals by FITC from the rDNAs followed the procedure described by Fukui *et al.* (1994 b). Images of chromosomes and signal regions of the locus were separately extracted from the respective G- and B-light images captured by a cooled CCD camera (Photometrics) and frozen in the memories of a personal computer. The G-and B-light images of the same chromosomal plate were combined into a single image.

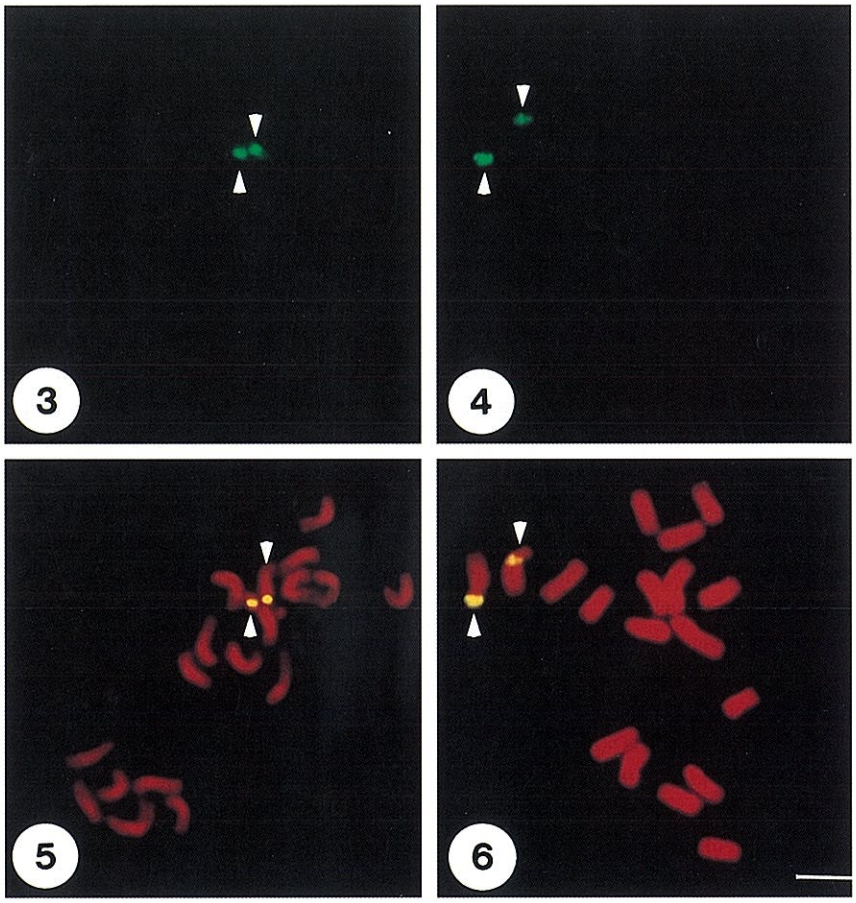
Total DNA was extracted from fresh leaves using cetyltrimethylammonium bromide, CTAB, and used for the RAPD analysis (Murray and Thompson 1980, Williams *et al.* 1990). The DNA from three individuals from each population was amplified using seven oligonucleotide primers (10 mer-kits, Operon Technologies, Inc., CA, USA) ; OPE 01: CCCAAGGTCC, OPE 02: G GTGCGGGAA, OPE 04: GTGACATGCC, OPE 11: GAGTCTCAGG, OPE 19: ACGGCGTATG, OPF 07: CCGATATCCC, and OPF 09: CCAAG CTTCC. Amplificating reactions were performed in a 25 μl volume, containing 20 ng of DNA, 1.5 mM $MgCl_2$, 0.4 mM of primer, 100 mM of dNTP (Pharmacia Biotech, USA), 1 U of Taq polymerase, and 1 x Taq polymerase buffer (Promega Co., USA). A thermal cycler, TSR-300, (Iwaki Co. Ltd., Japan) was used for the amplification. The cycling regimes consisting of the following steps: 40 cycles of 92℃ for 1 min, 38℃ for 1 min, and 72℃ for 2 min, with a final step of 72 ℃ for 7 min. The PCR products were fractionated by agarose gel electrophoresis and detected by staining with ethidium bromide solution.

Polymorphic bands obtained by the RAPD procedure were analyzed using a phenetic method. The binary data which is the absence or presence of bands was analysed by the simple maching coefficients (Sokal and Michener 1985). The distances $((d_1\ (a, b))^2=\sum_{j=1}^{b}(X_{aj}-X_{bj})^2)$ were culculated and a dendrogram was constructed using the unweighted pair-group methods with arithmetic averages, UPGMA, clustering algorithms (Sneath and Sokal 1973).

Results

Mitotic metaphase and prometaphase plates of *S. virgaurea* ssp. *asiatica* are shown (Figs. 1 and 2). The chromosome number was 2 n=18. Arrowheads in Figs. 1 and 2 indicate the nucleolar organizing regions (NORs) of the satellite-chromosomes. Relative length and arm ratio of each chromosome of a cell of *S. virgaurea* ssp. *asiatica* are shown in Table 2. The genome of *S. virgaurea* ssp. *asiatica* consisted of a satellite chromosome, a submetacentric chromosome, and seven metacentric chromosomes. The nomenclature for centromeric position of chromosomes depends on Levan *et al.* (1965). Abe and Takasu (1983) reported that the karyotype of *S. virgaurea* was K (2 n) =18=2 Am+2 Bsm+10 Cm+2 smDst+2 Em. They found some plants carrying two chromosomes which were difficult to make a pair by their chromosomal parameters. The karyotype we report here is very similar to that in the previous reports (Hujiwara 1962; Kapoor and Beaudry 1966; Abe and Takasu 1983). The genome of *S. virgaurea* ssp. *leiocarpa* consists of two submetacentric chromosomes with and without satellites and seven metacentric chromosomes (Nishikawa 1979, 1988). There seemed to





Figs. 3–6 : In situ hybridization of *Solidago* chromosomes. 3, G-light excitation image of a chromosomal plate of *S. virgaurea* ssp. *asiatica*. 4, G-light excitation image of a chromosomal plate of S. *virgaurea* ssp. *leiocarpa*. 5, Intergrated image of *S. virgaurea* ssp. *asiatica* obtained by image manipulation. 6, Intergrated image of *S. virgaurea* ssp. *leiocarpa* obtained by image manipulation. Arrowheads indicate satellite chromosomes. Bar indicates 5 μm.

Table 2. Relative length and arm ratio of each chromosome of *Solidago. virgaurea* ssp. *asiatica*

Chromosome number	Relative length of chromosomes	Arm ratio (long/short)
1*	5.93	4.15
2*	6.81	5.13
3	6.44	2.78
4	6.67	2.46
5	6.89	1.07
6	5.56	1.03
7	6.07	1.05
8	6.22	1.21
9	5.41	1.15
10	5.41	1.15
11	5.41	1.15
12	5.11	1.23
13	5.41	1.52
14	4.96	1.68
15	4.81	2.42
16	4.37	1.68
17	4.52	1.10
18	4.22	1.04
	total 100	

*Chromosomes 1 and 2 are chromosomes with satellites.

be no distinguishable differentiation between the karyotypes of *S. virgaurea* ssp. *asiatica* and *S. virgaurea* ssp. *leiocarpa*.

FISH revealed a single 45 S rDNA locus located on the satellite chromosome of *S. virgaurea*. Figures 3 and 4 show the G-light excitation image of chromosomal plates of *S. virgaurea* ssp. *asiatica* and *S. virgaurea* ssp. *leiocarpa*, respectively. White arrowheads indicate satellite-chromosomes which have nucleolar organizing regions (NORs). A pair of fluorescent signals of the 45 S rDNA locus was observed at the NORs on the same chromosomal plates of each subspecies (Figs. 5 and 6).

Figure 7 shows the PCR products amplified by primer OPF 09. Seven primers produced 71 bands, and 67 polymorphic bands were scored. The average number of bands per primer was 11.7. The molecular weights of the bands were from about 30 bp to 500 bp. Table 3 shows means of the genetic distances between individuals based on the polymorphism of the bands. A phenogram generated by an unweighted pair-group method with arithmetrical averages (UPGMA) is shown (Fig. 8). Except in one case (ON 23–ON 24), the three plants from single locations did not belong to the same group. Plants of the *asiatica*-, intermediate-, and *leiocarpa*-types, which were based on the gross morphology, dispersed in the different branches within the phenogram, although five plants (MY 20, ON 23, ON 24, MY 21, and ON 22) were on a branch. Except in one case (ON 23–ON 24), the three plants from single locations did not belong to the same group.

Discussion

Recently, variability in the 45 S rDNA locus was reported in the genus *Oryza* (Fukui *et al.* 1994 b). Variability in the number of the 45 S rDNA loci was observed between species and even between subspecies. For example, *O. glumaepatula* and *O. australiensis* have one and two rDNA loci, respectively. Within a species *O. sativa* L., ssp. *japonica* and ssp. *indica* possess one and two rDNA loci, respectively. The chromosomal structure of these species is considered to have changed during divergence of the sub-

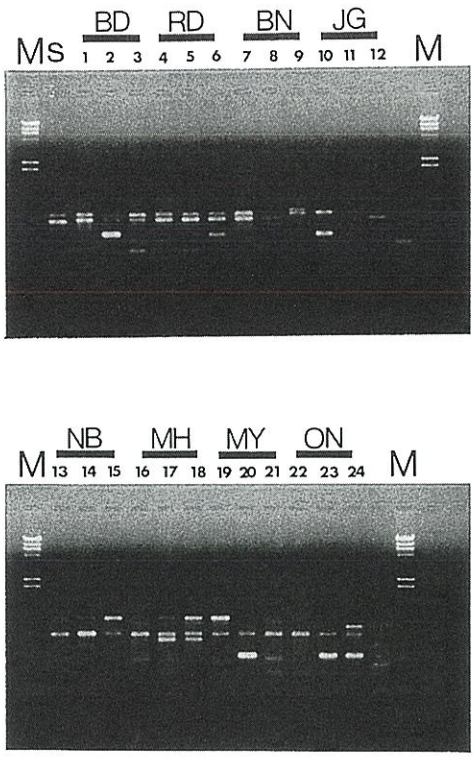


Fig. 7 : An agarose gel showing the PCR products which were amplified by using a decanucleotide primer : OPF 09. Lane M is the molecular size marker (λ / Hind III). Lane S is the control which was amplified from the genomic DNA of *S. altissima*. BD, RD, BN, JG, NB, MH, MY, and ON are the abbreviations of the localities. Numbers 1 to 24 are the numbers of samples.

species. In this investigation, *S. virgaurea* ssp. *asiatica* and ssp. *leiocarpa* were very similar in karyotype and the number and position of the 45 S rDNA locus, and were indistinguishable. There was no differentiation in visible chromosomal structures between the two subspecies studied.

Recent advances is the polymerase chain reaction (PCR) techniques provide sensitive methods for the analysis of DNA polymorphisms, such as RAPD analysis (Heun 1994 ; Abo-elwafa *et al.* 1995). Sixty-seven clear polymorphic bands were detected in RAPD analysis in this study. Other four bands appeared in all the samples examined. Some were specific to individuals and others were specific to populations. However, analysis resulting in a phenogram (Fig. 8) indicated that there was no relationship among RAPD banding patterns, altitude of sampling sites, and subspecies of *Solidago* based on morphological criteria. Individuals of typical *S. virgaurea* ssp. *asiatica* based on morphology did not all belong to the same branch, and the typical *S. virgaurea* ssp. *leiocarpa* based on morphology showed the similar tendency. Each individual of *S. virgaurea* ssp. *asiatica*, *S. virgaurea* ssp. *leiocarpa*, and intermediate types between two subspecies were dispersed apparently, randomly, on the phenogram. These results suggest there is no genetic isolation between *S. virgaurea* ssp. *asiatica*,

Table 3. Means of genetic distances between individuals. BD, RD, BN, JG, NB, MH, MY, and ON on the right side are the abbreviations of the sampling localities. Numbers 1 to 24 are the numbers of samples

	BD1	BD2	BD3	RD4	RD5	RD6	BN7	BN8	BN9	JG10	JG11	JG12	NB13	NB14	NB15	MH16	MH17	MH18	MY19	MY20	MY21	ON22	ON23	ON24
	5.000	5.745	5.916	5.568	4.899	5.099	5.831	5.831	5.292	5.916	5.000	5.000	5.477	5.292	5.292	6.000	5.916	5.292	5.657	5.568	5.657	6.164	5.568	5.831
BD1	−	3.464	4.000	4.243	3.317	3.000	4.123	3.873	3.317	4.000	3.464	3.162	4.796	4.123	3.606	4.359	4.243	3.317	4.583	3.742	4.123	4.583	4.000	4.359
BD2	−	−	4.000	4.472	4.123	3.873	4.359	4.359	4.123	3.464	4.000	4.000	5.000	4.359	4.123	4.796	4.472	3.606	4.583	3.742	4.359	4.583	4.000	4.123
BD3	−	−	−	3.742	4.123	4.359	4.583	4.796	4.123	4.243	4.899	4.690	4.796	4.359	4.359	3.606	4.000	4.359	4.123	4.472	4.123	3.606	4.243	4.583
RD4	−	−	−	−	3.606	3.317	4.123	4.583	3.873	4.243	4.243	4.000	4.123	3.000	3.873	3.873	3.742	3.606	3.873	4.243	4.123	3.873	4.690	5.000
RD5	−	−	−	−	−	3.464	3.742	4.243	3.742	4.123	4.123	4.123	4.000	4.000	4.000	4.000	3.317	3.162	4.000	4.123	3.464	4.243	3.873	4.000
RD6	−	−	−	−	−	−	4.243	4.472	3.464	4.123	3.317	3.317	4.690	3.464	3.742	4.243	4.359	3.464	4.472	3.873	4.000	4.690	4.123	4.690
BN7	−	−	−	−	−	−	−	3.742	4.243	4.359	4.583	4.796	5.099	4.243	4.243	4.243	3.606	3.742	4.243	4.359	4.243	4.243	4.359	4.243
BN8	−	−	−	−	−	−	−	−	4.243	4.796	4.583	4.583	5.099	4.690	4.472	4.690	4.123	4.000	4.690	4.359	4.690	4.472	4.123	4.243
BN9	−	−	−	−	−	−	−	−	−	3.606	3.873	4.359	4.690	3.742	3.742	4.000	4.123	3.742	4.472	4.359	3.742	4.000	4.123	4.000
JG10	−	−	−	−	−	−	−	−	−	−	4.243	4.690	4.796	4.359	4.359	4.000	4.123	4.583	3.742	4.123	4.123	4.123	3.742	3.873
JG11	−	−	−	−	−	−	−	−	−	−	−	3.162	4.123	3.873	3.606	4.359	4.690	3.873	4.583	3.742	4.359	5.000	4.243	4.796
JG12	−	−	−	−	−	−	−	−	−	−	−	−	4.359	3.873	3.873	4.583	4.899	3.606	4.583	3.464	4.583	5.000	4.243	4.796
NB13	−	−	−	−	−	−	−	−	−	−	−	−	−	4.243	4.243	3.742	4.796	4.690	4.243	4.123	4.243	4.472	4.123	4.690
NB14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	3.742	3.742	4.359	3.742	4.000	4.123	4.472	4.243	4.583	4.899
NB15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	4.243	4.123	3.464	4.000	3.606	3.742	4.000	3.873	4.243
MH16	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	3.873	4.000	4.000	3.873	4.000	4.000	4.123	4.690
MH17	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	3.606	4.359	4.472	3.873	3.606	4.243	4.123
MH18	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	4.472	3.873	4.000	4.472	4.359	4.472
MY19	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	3.873	4.243	4.000	4.123	4.690
MY20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	4.123	4.359	2.828	3.606
MY21	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2.828	3.317	4.000
ON22	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	3.606	4.000
ON23	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	2.236

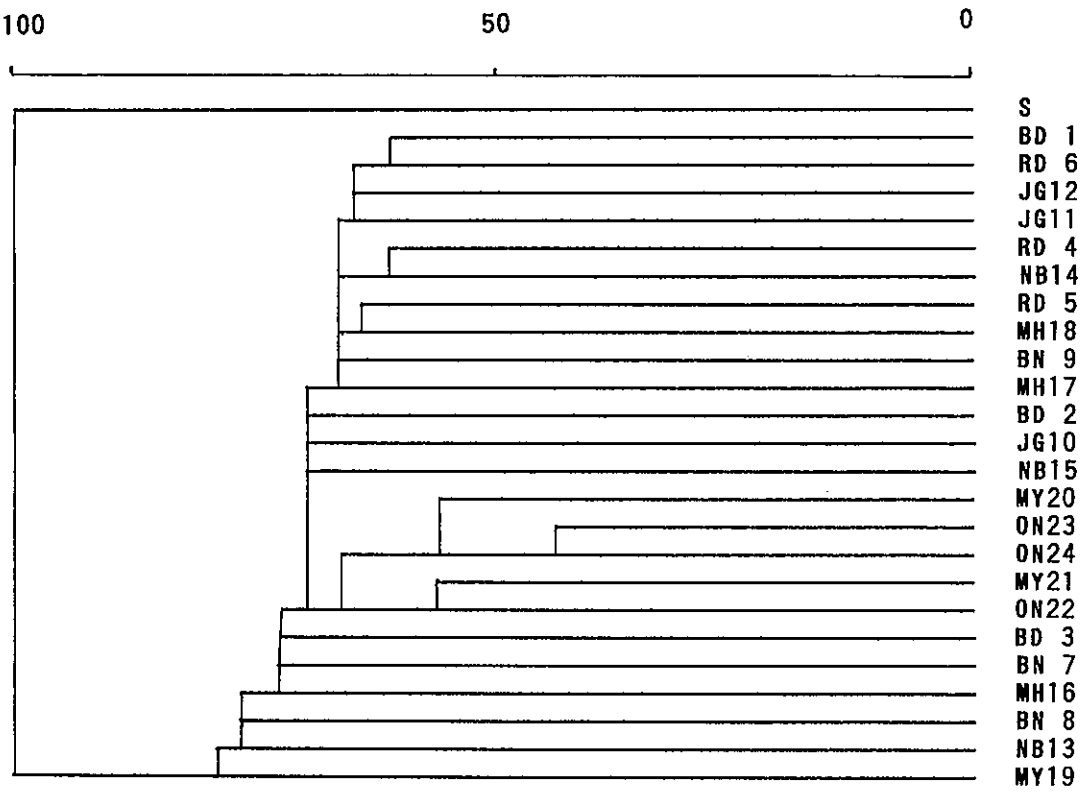


Fig. 8 : A phenogram generated using UPGMA. S shows a control, *S. altissima*. BD, RD, BN, JG, NB, MH, MY, and ON are the abbreviations of the sampling localities. Numbers 1 to 24 are the numbers of samples. Numbers 1, 50, 100 indicate the genetic distance.

S. virgaurea ssp. *leiocarpa* on Mt. Hakusan. The two subspecies could not be distinguished by the molecular methods currently employed. In conclusion, the data presented does not support differentiation of *S. virgaurea* into the two subspecies, ssp. *asiatica* and ssp. *leiocarpa* on Mt. Hakusan. Our results question the validity of separating *S. virgaurea* into ssp. *asiatica* and ssp. *leiocarpa* on Mt. Hakusan. Futher experimentation is needed to determine whether the morphological variation observed, when these subspecies grow in their natural habitat, is retained when grown in a similar environment.

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摘　要

広義のアキノキリンソウ *Solidago virgaurea* はヨーロッパからアジアまで分布するキク科植物である。日本列島には狭義のアキノキリンソウ *Solidago virgaurea* ssp. *asiatica*，ミヤマアキノキリンソウ（コガネギク）ssp. *leiocarpa* Hultén，キリガミネアキノキリンソウ f. *paludosa*（Honda）Kitamura，ハチジョウアキノキリンソウ var. *preaflorens* Nakai，オオアキノキリンソウ ssp. *gigantea*（Nakai）Kitamura，シマコガネギク var. *insularis* Kitamura などがあり，本州中部では狭義のアキノキリンソウは平地から亜高山帯まで分布しミヤマアキノキリンソウは高山帯に分布しているが，標高1600 m から 2500 m 付近には両者の中間的な個体があるという報告がなされている。本研究では石川県白山において狭義のアキノキリンソウとミヤマアキノキリンソウの間の染色体レベルあるいは分子レベルの変異を蛍光 in situ ハイブリダイゼーション（FISH）によるリボソーム遺伝子（rDNA）座の検出を含む染色体解析とランダムアンプリファイドポリモルフィック DNA（RAPD）分析を用いて調べた。

白山南西斜面の 8 集団から 3 個体ずつ計 24 個体を採集した。別当出合（標高 1260 m），中飯場林道（1520 m）から得られた個体はアキノキリンソウ型，弥陀ケ原（2350 m），水屋尻（2450 m），大汝峰（2670 m）から得られた個体はミヤマアキノキリンソウ型，別当覗（1810 m），甚之助小屋（1980 m），南竜馬場（2080 m）から得られた個体は中間的な外部形態を示した。体細胞染色体数はいずれも 2 n=18 で外部形態に変異が見られる個体間でも核型には相違が見られなかった。また核 DNA から PCR 法によりクローニングした 45 S rRNA 遺伝子をプローブとする FISH を行ったところ，いずれの分類群でも 1 対 2 個のシグナルが現れた。次に核 DNA からランダムプライマーを用いて増幅した産物の電気泳動パターンを調べた。その結果に基づき個体のグルーピングを試みたところ，外部形態上アキノキリンソウ型，中間型，ミヤマアキノキリンソウ型の個体がそれぞれグループを作ることなく分散した。以上から白山でのアキノキリンソウの 2 亜種：狭義のアキノキリンソウとミヤマアキノキリンソウの分布境界付近では外部形態だけでなく染色体や分子レベルの形質においても境界が不明瞭であることが明らかになった。

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