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RESEARCH STRATEGIES FOR FORAGE LEGUME BREEDING IN JAPAN

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Hokkaido is located in the northernmost part of Japan, extending from about 41 to 46 degrees latitude north, surrounded on all sides by oceans (Fig. 1). In comparison with main islands of Japan, climate in Hokkaido is dry and cool in summer and cold and snowy in winter. Cool climate and vast land area have made dairy farming to be one of the most important industries here. The pasture area in Japan is 0.64 million ha and that of Hokkaido is 0.53 million ha. More than 80 % of the pasture area is in Hokkaido. This is a reason why we have concentrated the breeding programs of forage legumes and grasses for this area.

We divide Hokkaido into main three areas, northern, central and southern, and eastern, based on meteorology, especially snow fall (Fig. 1, Table 1). The northern area has heavy snow in winter, sunny and less precipitation in spring and cool and wet in summer. The eastern area is less snow fall and severe soil frost. The central and southern area shows mild through the year and much snow fall in winter. In Hokkaido the most important breeding objective is improvement of winter hardiness. We usually separate Hokkaido to two areas in winter condition, heavy snow and severe cold with less snow. In heavy snow area forage plants are damaged by snow mold and in less snowy area severe cold damages plants by freezing. We have improved resistance of cultivars to both damages. In addition, we usually grow forage legumes mixed with grass species, timothy and orchard grass. There are some difficulties to maintain appropriate legume percentage, caused by competition among grass and legume species. The compatibility of mixed sown is another important objective for forage legume breeding.

Fig. 1. Three main areas in Hokkaido based on the climatic condition.

Development of new cultivars of forage legumes

Breeding of red clover. More than one hundred thirty years have past since we firstly introduced forage seeds from the United States and started evaluation of their performance. Forty years later we established recommended variety system as forage, including red clover and alfalfa, as the result of the performance tests at the local stations. At the same time cultures of red clover as green manure for crop rotation were expanded. The growth of culture area of red clover through Hokkaido and the following natural and artificial selection made ecotypes and landraces which have been used as main breeding materials for new cultivars (Fig. 2).

In the early days of breeding the most important objectives were yield and persistency because most of introduced varieties did not

Table 1

Climate statistics of representative cities in each area in Hokkaido

Area	City	Mean temperature (C°)			Snow Depth (cm)	Precipitation (mm)			Frost free days
		Year	Max. in Aug	Min. in Feb		May to Oct	Nov to Apr	Year	
North	1 Wakkanai	6.4	22.0	–8.2	90	597	527	1,124	184
East	2 Kushiro	5.7	21.3	–11.6	28	697	345	1,043	156
East	3 Obihiro	6.3	24.9	–13.8	49	610	307	917	142
Center	4 Sappor	8.2	26.1	–8.0	95	585	544	1,130	172
South	5 Hakodate	8.5	25.5	–7.2	40	701	454	1,155	160

have enough adaptability in this areas and showed low persistency. In 1966 two domestic varieties «Sapporo» and «Hamidori» were developed. Main breeding materials were selected plants from Hokkaido land races. Both cultivars showed superior yield and persistency than introduced ones. Then in 1989 we developed new cultivars, «Hokseki» and «Taisetsu». «Hokuseki» is a diploid cultivar bred with polycross of six maternal lines, which were selected from «Sapporo» based on the yield of 3rd year and seed productivity (Table 2, Fig. 3). Taisetsu is a tetraploid cultivar bred with polycross of ten maternal lines which were selected from «Sapporo» and doubled chromosome number by dinitrogen oxide. Nowadays «Hokuseki» is a leading variety in Hokkaido. In contrast, «Taisetsu» is not utilized, because of low seed productivity and too much competitive ability to grasses. For further improvement of persistency and compatibility with grasses, especially timothy, we developed a new cultivar «Natsuyu» in 2001. «Natsuyu» shows low competitive ability for timothy and superior over-wintering.

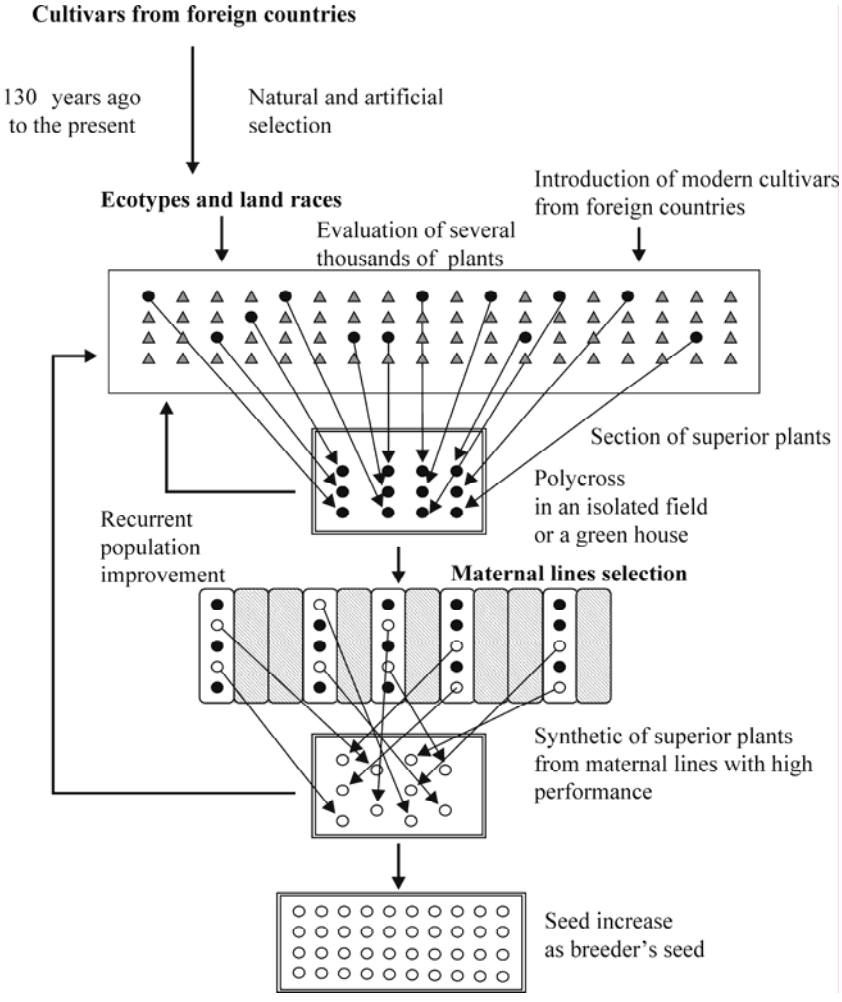

Fig. 2. Modified maternal line selection for red clover at NARCH.

Our main breeding method for red clover is maternal line selection and polycross among plants from selected line (Fig. 2). We are im-

Table 2

Varieties developed at NARCH and their breeding

	Sapporo	Hokuseki	Taisetsu	Natsuyu
Registered year	1966	1989	1989	2001
Ploidy level	Di	Di	Tetra	Di
Breeding method	Mass selection	Maternal line selection	Synthetic variety	Maternal line selection
Materials	Land races	Sapporo	Sapporo	Introduced cultivars and Sapporo landraces
Breeding objecitives	Yield and persistency	Yield and persistency	Yield and persistency	Mixed sown with timothy

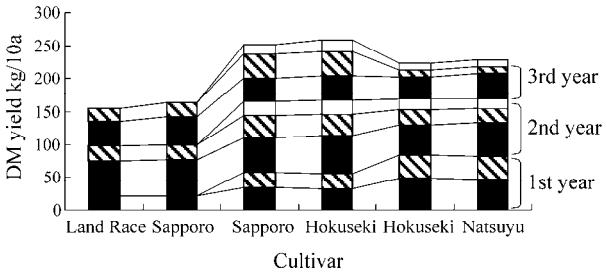

Fig. 3. Comparison of DM yield between a new cultivar and standard cultivar in red clover cultivars of different registered year.
Registration year, Sapporo in 1966, Hokuseki in 1983, Natsuyu in 2001.

proving persistency and winter hardiness of new lines. In addition to early flowering variety we are developing very early and late flowering lines for diverse production system in this area.

Breeding of white clover. White clover is quite new for our breeding programs, we just started it in 2001. Now we are evaluating and selecting the plants with winter hardiness among genetic resources introduced from the gene banks and other research institutes.

Breeding of alfalfa. We developed a first cultivar of alfalfa, «Kitawakaba», which adapted for Hokkaido region in 1983. However, Verticillium wilt spread through this region after the release of «Kitawakaba». «Kitawakaba» was severely damaged by this disease. «Kitawakaba» also was not adaptable in the eastern area of Hokkaido according to severe winter cold with less snow. Then we improved these traits by introduction of foreign germplasm and selection, and developed two cultivars, «Makiwakaba» and «Hisawakaba» in 1994 (Fig. 4). «Makiwakaba» is tolerant to Verticillium wilt and has lodging resistance. «Makiwakaba» is recommended for cultivation in snowy areas of Hokkaido and northern Tohoku. «Hisawakaba» is more resistant to common leaf spot caused by *Pseudopeziza medicaginis* and Leptosphaerulina leaf spot caused by *Leptosphaerulina briosiana* than «Kitawakaba». «Hisawakaba» is recommended for cultivation in less snowy areas of Hokkaido.

In 2003 we have just developed a new cultivar, «Haruwakaba». «Haruwakaba» has more persistent and stable productivity than «Makiwakaba» and «Hisawakaba» due to its high winter hardiness. Disease tolerance of «Haruwakaba» is almost same level as «Makiwakaba» and «Hisawakaba».

Our main breeding method for alfalfa is synthetic variety and maternal line selection. After spread of Verticillium wilt we developed an inoculation method for estimating the level of resistance of cultivars to Verticillium wilt (Sato, 1985). Now we are developing lines with highly lodging resistance and trampling tolerance for bigger machinery harvest.

Genome analysis and development of DNA markers for forage legume breeding

RFLP linkage map of red clover. Most of agronomical important characteristics are controlled by quantitative traits loci (QTLs), it is difficult to improve them efficiently by conventional selection methods. Linkage maps using DNA markers help us to identify QTLs linked to traits of agronomical importance and can be applied to marker-assisted selection.

We constructed a first genetic linkage map of red clover using RFLP markers from cDNA prob-

es of a backcrossed mapping population (Fig. 5), and investigated the transferability of the markers to other red clover germplasms (Isobe *et al*., 2003). The map contains 157 RFLP markers and one morphological marker on seven linkage groups. The total map distance was 535.7 cM and the average distance between two markers was 3.4 cM. All of the cDNA probes of the map were hybridized to the fragments of genomic DNA from 12 plants derived from three varieties, and 87 % of the cDNA probes detected polymorphic bands that corresponded to those of mapping parents.

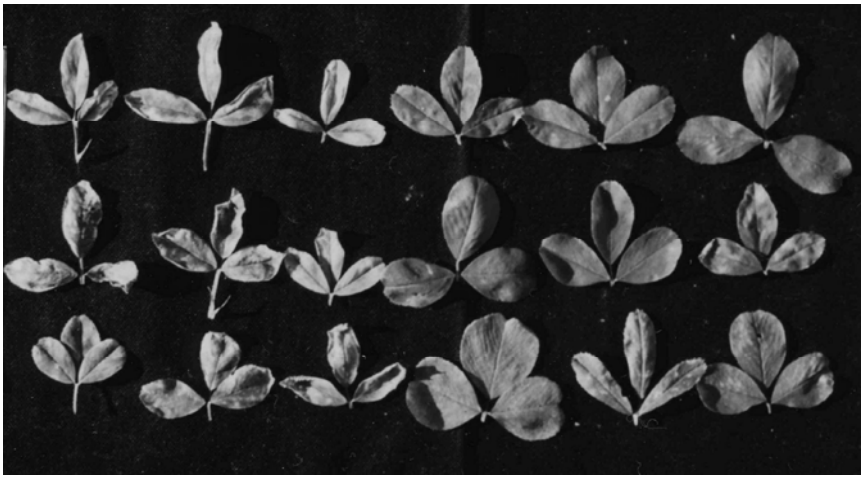

Fig. 4. Symptom of Verticillium wilt on leaves of a susceptible variety, Kitawakaba (left) and a resistant variety Hisawakada (right).

Development of PCR-based markers and construction of high density maps. One hundred and thirty five cDNA probes of RFLP markers were sequenced and annotated. The red clover cDNA sequences showed high similarity to model legume plants, *Medicago truncatula* and *Lotus japonicus*. We are trying to develop transform the RFLP markers to PCR-based markers. We are also constructing a high density red clover linkage map with EST-SSR markers using EST data of red clover and *Lotus japonicus*. Our goal is to construct a map by 1500-2000 markers.

QTL analysis of Sclerotinia tolerance and flowering time. Sclerotinia root rot and crown rot, caused by the fungus *Sclerotinia trifoliorum* Erickson, is one of the most destructive diseases in Hokkaido. Flowering time is also an important trait, since it is related to compatibility of grass species. Putative QTLs of Sclerotinia tolerance and flowering time were detected based on the data of the mapping popula-

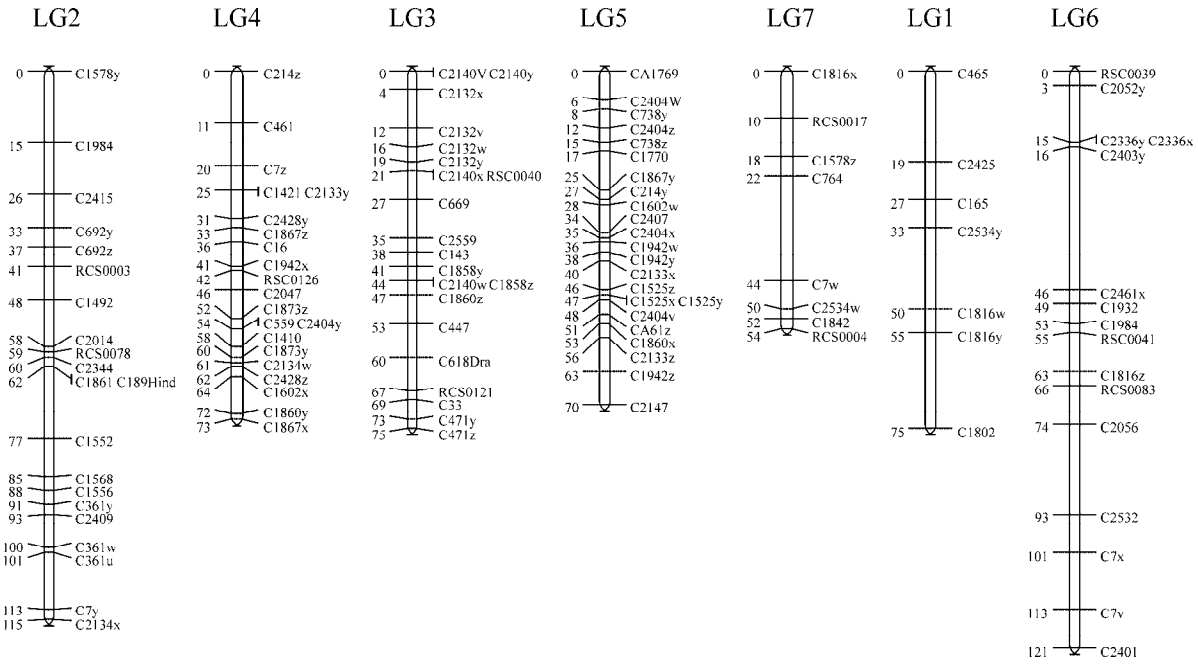

Fig. 5. Linkage map of red clover based on 157 RFLP markers.

tion. Several QTLs of Sclerotinia tolerance were detected near by the candidate genes those encode cold or stress tolerance (Klimenko *et al*., 2003). This work is part of an ongoing joint research project among NARCH, All-Russian Williams Fodder Crop Research Institute (Russia) and Kazusa DNA research institute (Japan).

Interspecific hybridization. Red clover is a short lived perennial species, it disappears by the fourth year of use. It is grown in mixtures with perennial grasses, such as timothy, which are long lived and usually used more than five years. The improvement of persistency is one of the most important objectives for red clover breeding, since decrease of legume percentage causes nutritional deterioration of products from pastures.

Zigzag clover (*Trifolium medium* L., $2n = 80$) is a relative species of red clover, extremely perennial and also propagates vegetatively by rhizomes. We have applied interspecific hybridization between red clover and zigzag clover for the improvement of persistency. Hybrid plants were obtained through embryo cultures, only nine plants ($2n = 50$) of 217 immature embryos were successfully grown to maturity (Sawai *et al*., 1990). Then these plants were backcrossed with red clover, and three of them showed partial female fertility, and one plants produced rhizomes. After the chromosome doubling further backcrosses had been done (Sawai *et al*., 1995). The pollen fertilities restored from 0–4.6 % at BC1 and to 2.3–36.8 % at BC2.

The backcross progenies increased both female and pollen fertility as the backcross generation advanced. Female and pollen fertility of BC4 plants 21 and 65 %, respectively (Fig. 6) and produced mature seeds by honey bees in the field condition (Isobe *et al*., 2002). The BC4 plants were transplanted on the filed and selected for persistency. Then, the selected BC4 plants were cross pollinated and the BC4F1 progenies were selected for seed productivity. Now the BC4F2 progenies have been planted in the field and evaluated their characteristics, especially persistency.

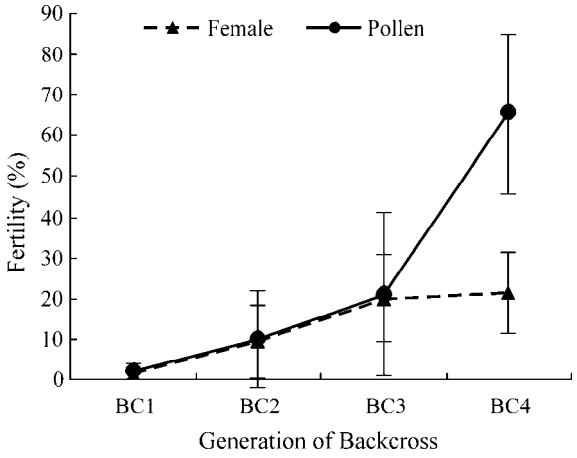

Fig. 6. Female and pollen fertility of the backcross progenies between red clover and zigzag clover.

Seed Multiplication and Field Evaluation of Miyako-gusa (*Lotus japonicus*)

Lotus japonicus is a wild perennial herbal legume with a small genome ($2n = 2x = 12$) and a short life cycle. This plant is expected to play a role as the model organism of leguminous plants including soybean and forage legumes. This project is supported by Japanese Government, Ministry of Education, Culture, Sports, Science and Technology and Miyazaki University is the center of this project. Under this project we are in charge of seed multiplication of the recombinant inbred lines (RI's) and evaluation of 108 RI's and 59 ecotypes collected through Japan. Now we are evaluating the traits including flowering and winter hardiness on the field and detecting QTLs using RI's.

New strategies for facilitating breeding programs of forage legumes

International collaborative research with Williams All-Russian Fodder Crop Research Institute. Williams All-Russian Fodder Research Institute (WFRI) and NARCH has developing collaborative relationships more than ten years. We have visited each side and exchange germplasms, and evaluated common materials at both sides. Now we have a collaborative program about QTL analysis of red clover winter hardiness under the International Joint Research for Improvement of Winter Wheat and Forages for Low-temperature Stress in Northern Area. In this project we are developing new breeding materials as follows.

We grow timothy in more than 80 % of pastures in Hokkaido. Timothy has good quality and cold tolerance, however, its regrowth after 1st cutting is poor. On the other hand, red clover shows excellent regrowth in summer. It

Table 3

Traits of selected plants, their original F2 population and the parental lines

Line/genotype	Spring vigor	Flowering date in spring	Plant vigor at 1st cutting	Flowers per a head	Flowering date in summer	Score of flowering stems
Hokuseki	3.7	6/27	4.3	4.8	8/6	3.5
F2	4.5	6/20	4.7	4.7	8/7	2.8
RANNY2	3.7	6/30	3.4	4.4	8/24	1.0
Selected plants						
F2-1-31	6	6/15	7	7	7/27	3
F2-9-25	3	6/16	4	4	8/10	2
F2-13-26	6	6/16	5	6	8/11	2
F2-14-23	6	6/17	6	4	8/13	1
F2-16-17	6	6/17	6	5		0
F2-18-19	7	6/17	7	6	8/10	1
Average of selected plants	5.7	6/16	5.8	5.3	8/8	1.5

Except for flowering date, each index was scored at 1 (low) to 9 (high) scale based on visual observation.

causes depress the regrowth of timothy and after red clover died naked area increases where weed species easily invade.

A Russian cultivar, «Ranny2» which was developed at WFRI, is early flowering in spring, but lower flowering ratio in summer. It has desirable characters for mixture with timothy, which Japanese red clover cultivars do not have. We developed a F_2 population from a cross between «Ranny2» and «Hokuseki», and selected superior plants which shows superior persistency, disease resistance and mild regrowth (Table 3).

Marker-assisted selection. We have now detected some QTLs affecting winter hardiness and flowering time in red clover. In addition to the construction of a high density map, we are planning of detection of QTL using a kind of linkage disequilibrium (LD) mapping. For the purpose of development of useful markers for breeding information in a map from a specific mapping population is not adequate because of lack of polymorphisms. We are growing a large population originated from genetically wide range of germplasms and directly analyzing the relationships between markers and traits, and further interaction among them. We are sure these markers accelerate our breeding program as marker-assisted selection.

New breeding objectives corresponding to the change of farming system. Recently the trends of the decrease of the number of dairy farmers and large scale farming make the number of cattle and the area of pasture per one farmer increase rapidly. In addition economic stagnation and aging of farmers cause large scale machinery, contractor harvest system and delay of pasture renovation. We must consider breeding objectives responding to the change of farmer's and society's background. We started new breeding programs, wheel traffic resistant alfalfa for big harvest machine, extremely early flowering and superior early growth red clover for re-seeding and over-seeding on old pasture.

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