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CYTOLOGICAL STUDIES OF SEVERAL INTERSPECIFIC F₁ HYBRIDS IN THE SUBGENUS *EUMELILOTUS*

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Introduction

The genus *Melilotus* (sweetclover) is comprised of some twenty species (SCHULZ 1901, ISELY 1954, STEVENSON 1969). Most of the cultivated varieties which have been used for forage and soil improvement for many years are derivatives of two species, *M. alba* and *M. officinalis*, belonging to the subgenus *Eumelilotus*.

Chlorophyll deficiency widely occurring in the interspecific hybrids within 9 species of the subgenus *Eumelilotus*, constitutes an effective barrier against the exchange of genes between the two species (SMITH 1954, KITA 1965).

Regarding cytological studies in the interspecific F₁ hybrids, several papers have been reported by several workers (WEBSTER 1950, 1955, BRINGHURST 1951, SHASTRY, SMITH and COOPER 1960, JARANOWSKI 1961, KITA 1962, 1965, KITA and NIIZEKI 1967). From the studies of meiotic chromosome behaviors of the interspecific F₁ hybrids, it appeared that structural differences of chromosomes were in a role of speciation of the genus *Melilotus* in part.

The purpose of this study is to make clear cytological relationships among species of the subgenus *Eumelilotus* systematically.

Materials and Methods

Eight species belonging to the subgenus *Eumelilotus* were used in this study. These are described in the results.

Cross pollinations were made in the green house from 1970 to 1972. In making each cross, the petals were removed from recently opened flowers and pollens were removed by suction using a small vacuum pump.

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Pollen of the staminate parent was applied without delay to the stigma of the pistillate parent with a toothpick tip (SMITH 1954).

Seeds obtained were classified by size and plumpness and seed coats were scratched with a razor blade, and then germinated in a petry dish under favorable temperatures. Germinated seeds were transferred to flats filled with soil. Most of the interspecific hybrids were easily detected by chlorophyll deficiency in the seedling stage and/or morphological differences from the maternal and paternal parent species, as described in the results.

Young inflorescences of the desired stage of development were fixed in a mixture of 3 parts absolute alcohol and 1 part glacial acetic acid to which was added a few drops of 45% glacial acetic acid saturated with ferric chloride, and maintained at low temperatures (ca. 2-4°C) for 24 hours. The anthers were removed and smeared in a drop of acetocarmine and the meiotic configurations were analysed.

Pollen grains from mature anthers were placed in a drop of acetocarmine in order to determine the percentage of stainable pollen grains.

Results

1. Cytological observations in the parental species

All of the parental strains of each species of the subgenus *Eumelilotus* used in this study had high pollen fertility, having higher than 90% except *M. polonica* C 30. The parental strains at each of the following stages in microsporogenesis -diakinesis, metaphase 1, anaphase 1, and anaphase 2- were examined. In all of the parental strains without exception, meiosis was normal with eight bivalents (8_{II}) regularly present at diakinesis and metaphase 1 (Fig. 1). Some abnormalities following irregular disjunctions such as a lagging chromosome were present in a very small proportion of the spore nuclei.

As reported by WEBSTER (1950), it is considered that high pollen sterility of *M. polonica* C 30 (ca. 0.0-21.4% of stainable pollen grains) was caused by some environmental factors such as high temperatures rather than chromosomal abnormalities. As far as examined microsporogenesis, meiosis was normal.

2. Cytological observations in the F₁ hybrids

a. *M. alba* × *M. suaveolens* F₁

In the crosses between *M. alba* and *M. suaveolens*, five biennial strains of *M. alba*, Arctic, Bdn 928, Bdn 647, Cumino, and Denta, were used as pistillate parents, and three biennial strains of *M. suaveolens*,

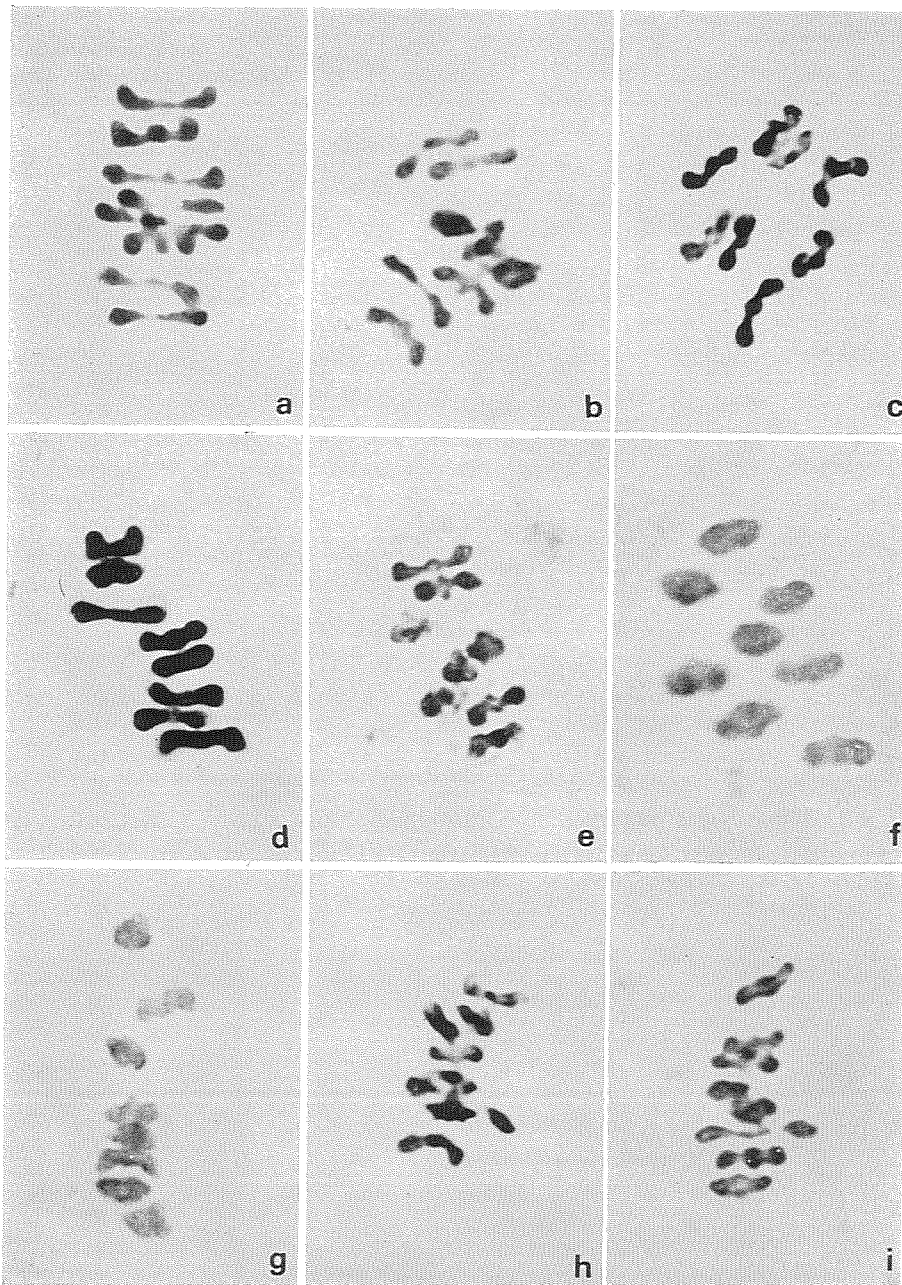


Fig. 1. Metaphase I of the parental species. $\times 2100$.

- a. *M. alba* Arctic b. *M. suaveolens* Redfield Yellow c. *M. officinalis* P.I. 178985 d. *M. officinalis* Gold Top e. *M. hirsuta* P.I. 314096
 f. *M. polonica* 3446 g. *M. taurica* 70 h. *M. dentata* Bdn 58-190
 i. *M. wolgica* 58-254

TABLE 1. Pollen fertility of the interspecific F₁ hybrids examined

	cross	no. of plants	average of pollen fertility (%)
<i>M. alba</i> Arctic	× <i>M. suaveolens</i> 70	2	28.2
" "	× " 18-4	5	26.8
" "	× " Golden Annual	11	40.1
" Cumino	× " 70	2	40.3
" Bdn 928	× " Redfield Yellow	4	53.0
" Denta	× " Bdn 60-59	1	19.1
" Denta	× " Redfield Yellow	1	28.7
<i>M. officinalis</i> P.I. 178985	× <i>M. alba</i> Arctic	4	52.4
" "	" × " Cumino	2	57.1
" "	" × " Brandon Dwarf	4	63.8
" "	" × " Bdn 928	10	51.0
" "	" × " Bdn 647	1	67.1
" "	" × " Hubam	2	57.1
" "	" × <i>M. hirsuta</i> P.I. 314096	11	96.8
" "	" × " 58-4	2	86.8
" "	" × <i>M. polonica</i> 3446	4	98.0
<i>M. polonica</i> C 30	× <i>M. suaveolens</i> Redfield Yellow	4	20.4
<i>M. hirsuta</i> 58-4	× " Bdn 60-59	1	87.6
<i>M. alba</i> Arctic	× <i>M. hirsuta</i> P.I. 314096	11	67.6
" Bdn 928	× " "	4	66.8
" Bdn 647	× " "	3	59.7
<i>M. hirsuta</i> 58-4	× <i>M. alba</i> Bdn 647	2	42.2
" P.I. 314096	× " "	1	60.0
" "	× " Hubam	2	50.4
<i>M. alba</i> Arctic	× <i>M. polonica</i> 3446	6	27.0
" Denta	× " "	12	56.1
" Bdn 928	× " "	5	60.0
" Hubam	× " "	2	56.3
<i>M. polonica</i> C 30	× <i>M. alba</i> Arctic	1	56.2
" "	× " Evergreen	2	34.8
" 3446	× " Arctic	3	44.9
" "	× " Hubam	1	59.9
<i>M. hirsuta</i> 58-4	× <i>M. polonica</i> 3446	8	96.4
<i>M. taurica</i> 70	× <i>M. alba</i> Cumino	6	25.0
" "	× " Denta	2	25.2
" P.I. 314094	× " Cumino	1	27.7
" 70	× <i>M. wolgica</i> 58-254	5	74.5
<i>M. dentata</i> Bdn 58-190	× <i>M. taurica</i> 70	2	41.7
" "	× " P.I. 314094	1	24.7
" P 783-2	× " 134-1	1	1.9

Redfield Yellow, 70, Bdn 60-59, and two annual strains of *M. suaveolens*, 18-4, Golden Annual, were used as pollen parents.

From these crosses, many seeds which consisted of plump, small, and brown aborted seeds were obtained. All of the successful hybrid seedlings,

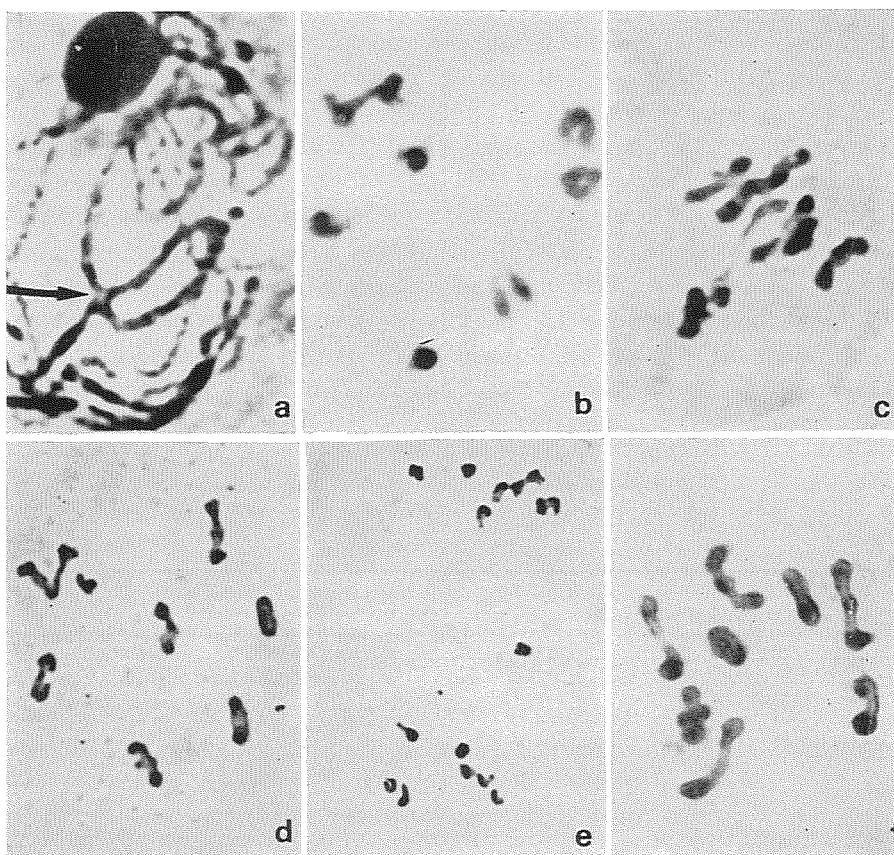


Fig. 2. Different stages in meiosis of the F_1 hybrids examined.

- a. *M. alba* Denta \times *M. suaveolens* Bdn 60-59 F_1 . Pachytene showing a cross-shaped configuration. The breakage point is indicated by arrow. $\times 2450$.
- b. *M. alba* Bdn 928 \times *M. suaveolens* Redfield Yellow F_1 . Diakinesis with 6II plus a chain of 4 chromosomes. $\times 1800$.
- c. The same F_1 as b. Metaphase I with 1IV + 6II. $\times 2100$.
- d. *M. officinalis* P.I. 178985 \times *M. alba* Bdn 928 F_1 . Metaphase I with 1III + 6II + 1I. $\times 1500$.
- e. The same F_1 as d. Anaphase I with a lagging chromosome. $\times 1300$.
- f. *M. officinalis* Gold Top \times *M. officinalis* P.I. 178985 F_1 . Metaphase I with 8II. $\times 2100$.

in the most of cases, were derived from the small seeds and grew vigorously. Upon flowering, all plants produced pale yellow flowers indicating that they were hybrids since the maternal parent, *M. alba*, was white-flowered. On the other hand, when annual strains of *M. alba* such as Hubam, Bdn 631, and Bdn 633, were used as pistillate parents, the F_1 hybrid seedlings showed moderate chlorophyll deficiency and were very weak. The seedlings were not able to survive beyond cotyledonary stage.

In the reciprocal crosses, *M. suaveolens* \times *M. alba*, by using the same strains of *M. alba* as pollen parents, a lot of aborted seeds were obtained, but no hybrid seedlings.

All of the hybrid plants obtained were highly sterile as shown in Table 1, and aberrant chromosome behaviors occurred during the course of meiosis. Usually, six bivalents and a chain of four chromosomes existed at diakinesis and metaphase 1 (Fig. 2 b, c). Six bivalents plus a trivalent and one univalent ($6_{II} + 1_{III} + 1_I$), seven bivalents plus two univalents ($7_{II} + 2_I$) and eight bivalents (8_{II}) were present in the remaining nuclei. High fre-

TABLE 2. Chromosome configurations at diakinesis and metaphase 1 and their distribution in later stages of meiosis in the interspecific F_1 hybrids, showing that they are heterozygous for a reciprocal translocation

cross	no. of cross combinations examined	stage	frequency of PMCs with						total
			$1_{IV} + 6_{II}$	$1_{III} + 6_{II} + 1_I$	8_{II}	$7_{II} + 2_I$	nor.	abnor.	
<i>M. alba</i> \times <i>M. suaveolens</i>	5	diakinesis	272	49	49	17			388
		metaphase 1	273	186	27	24			510
		anaphase 1					156	89	245
		anaphase 2					181	51	232
<i>M. officinalis</i> \times <i>M. alba</i>	4	diakinesis	268	40	40	29			377
		metaphase 1	227	218	31	33			509
		anaphase 1					202	103	305
		anaphase 2					239	73	311
<i>M. alba</i> \times <i>M. hirsuta</i> and the reciprocal	4	diakinesis	193	27	21	19			260
		metaphase 1	158	178	14	11			361
		anaphase 1					127	94	221
		anaphase 2					185	78	263
<i>M. alba</i> \times <i>M. polonica</i> and the reciprocal	3	diakinesis	67	11	12	4			94
		metaphase 1	64	94	17	18			193
		anaphase 1					55	26	79
		anaphase 2					77	23	100

quency of the chromosome association such as $6_{II}+1_{III}+1_I$ at metaphase 1, presumably, indicates a precocious segregation of one member of the chain. Pachytene analysis was undertaken in order to determine whether the multivalent association of four chromosomes was the result of a reciprocal translocation. A cross-shaped configuration observed in pachytene stage clearly revealed that the multivalent association of four chromosomes was of the reciprocal translocation origin (Fig. 2 a). Some abnormalities such as 7-9 disjunction which could lead to the production of aneuploid gametes, and lagging chromosomes occurred at anaphase 1. Lagging chromosomes were also present at anaphase 2, and the frequency of the abnormalities was relatively high.

From the observations mentioned above, it is clear that the F_1 hybrids between *M. alba* and *M. suaveolens* in all cross combinations examined, are heterozygous for a reciprocal translocation, explaining partial pollen fertility. Consequently, this result is different from WEBSTER's result (1950) that the course of meiosis in the F_1 hybrid between *M. alba* and *M. suaveolens* was normal.

Chromosome configurations at diakinesis, metaphas 1, and distributions of chromosomes at anaphase 1 and anaphase 2 in several hybrids are presented in Tabla 2.

b. *M. officinalis* × *M. alba* F_1

It has been generally considered that the two principal economic species, *M. alba* and *M. officinalis*, are completely isolated reproductively, since none of the varieties of either species has been known to hybridize with those of the other and produce a fully developed hybrid seed or mature F_1 plant without the use of embryo culture. However, it was reported that *M. officinalis* P.I. 178985, an introduction from Turkey, was crossed successfully with *M. alba* Spanish in natural conditions (LANG and GORZ 1960).

In this experiment, reciprocal crossings between *M. officinalis* P.I. 178985 and each of many strains of *M. alba* were made. When *M. officinalis* P.I. 178985 was used as the pistillate parent a lot of F_1 hybrids were obtained but no hybrid plant resulted from the reciprocal. The hybrid seedlings continued to grow to maturity in spite of having slight chlorophyll deficiency.

All hybrids obtained had partial pollen sterility (Table 1). Six bivalents and a chain of four chromosomes, as shown in Table 2, frequently occurred at diakinesis and metaphase 1 of microsporogenesis in all cross combinations (Fig. 2 d.) Cross-shaped configurations characteristics of a

reciprocal translocation were present at pachytene, indicating that one of the parent is homozygous for an interchange of relatively large section between two unhomologous chromosomes as well as in the case of the hybrid of *M. alba* × *M. suaveolens*. At anaphase 1 and anaphase 2, irregularities in chromosome disjunction such as lagging chromosomes existed to some extent (Fig. 2 e).

The observations described above are different from the result reported by SHASTRY *et al* (1960) that meiotic chromosome behaviors in the F₁ hybrid between *M. alba* and *M. officinalis*, which was reared by using embryo culture (WEBSTER 1955), were regular.

In order to resolve the controversial result, it became necessary that the intraspecific F₁ hybrids between P.I. 178985 and the other strains of *M. officinalis* should be cytologically examined. Since no chromosomal difference apparently existed among the strains of *M. alba*, if the both results were true, the chromosomal difference might be observed in the interstrain F₁ hybrids between P.I. 178985 and the other strains of *M. officinalis*.

c. The intraspecific F₁ hybrids between P.I. 178985 and the other strains of *M. officinalis*.

The reciprocal crossings between P.I. 178985 and the other eight strains of *M. officinalis*, Bdn 62-1, Goldtop, Madrid, Bdn 62-13, Erector, P 197-1, P 198-1 and P 200-2, were made. The F₁ plants from the crosses in which P.I. 178985 was used as pollen parent, were normally green and produced large pods characteristic of P.I. 178985, showing that they were F₁ hybrids. Although the F₁ plants resulted from the reciprocal crosses using P.I. 178985 as pistil parents could not be identified with the certainty by morphological characteristics whether they were F₁ hybrids

TABLE 3. Chromosome configurations at diakinesis and metaphase 1 and their distribution in later stages of meiosis in the intraspecific F₁ hybrids of *M. officinalis*, showing that meiotic chromosome behaviors were regular

no. of cross combinations examined	stage	frequency of PMCs with				total
		8II	7II+2I	normal	abnormal	
6	diakinesis	192	10			202
	metaphase 1	209	39			248
	anaphase 1			153	3	156
	anaphase 2			77	2	79

or not, the most of crossed plants were also considered as the successful hybrids. The reasons for this are that crossability was very high in the crosses in spite of the very high degree of self incompatibility in all strains of *M. officinalis*. The intraspecific F_1 plants made good growth as well as the parental strains.

The pollen fertility of the F_1 hybrids was higher than 90%. And the chromosomal behaviors at meiosis were remarkably regular in all cross combinations of F_1 hybrids, namely, the chromosome association such as 8_{II} occurred constantly at diakinesis and metaphase 1 (Fig. 2 f); and normal disjunction was present in most of microspore nuclei at anaphase 1 and anaphase 2 (Table 3). As far as examined, it was considered that there was no chromosomal difference among these strains of *M. officinalis*. Henceforce, it is reasonably concluded that structural differences of chromosomes by a reciprocal translocation exist between *M. alba* and *M. officinalis*.

d. *M. officinalis* P.I. 178985 \times *M. hirsuta* F_1

M. officinalis P.I. 178985 crossed readily not only with *M. alba* but also with two strains of *M. hirsuta* P.I. 314096 and 58-4. The hybrid seedlings from greenish small seeds were distinctly chlorophyll deficient, but as the growth progressed the leaves gradually increased green. In the reciprocal crosses, *M. hirsuta* \times *M. officinalis* P.I. 178985, by using the same strains of *M. hirsuta* as pistillate parents, numerous cross pollinations were made in vain. All F_1 hybrids obtained had high pollen fertility (Table 1). As being expected from high pollen fertility, chromosome associations at diakinesis and metaphase 1 indicated a high degree of homology between the chromosomes of the two species. Usually, eight bivalents (8_{II}) were present at diakinesis and metaphase 1 (Fig. 3 a, b), while in the remainder, seven bivalents plus two univalents ($7_{II} + 2_I$) occurred. Abnormal disjunctions in later stages of meiosis were quite rare. Chromosome configurations at diakinesis and metaphase 1, and their distributions in later stages of meiosis are given in Table 4.

e. *M. officinalis* P.I. 178985 \times *M. polonica* F_1

M. officinalis P.I. 178985 was also able to cross readily with *M. polonica* 3446, when *M. officinalis* P.I. 178985 was used as the pistillate parent in the cross, and many small F_1 seeds were obtained. All seedlings germinated from these seeds did not show chlorophyll deficiency at all and were vigorous, being almost as strong as the parental species. Margin of their leaflets was sharply dentate and the plants produced lax racemes

showing characteristic of *M. polonica*. The reciprocal cross, *M. polonica* 3446 \times *M. officinalis* P.I. 178985, was also attempted but no hybrid seedling was obtained, though many flowers were cross pollinated.

The F_1 hybrids had high pollen fertility, having about 97.9% of

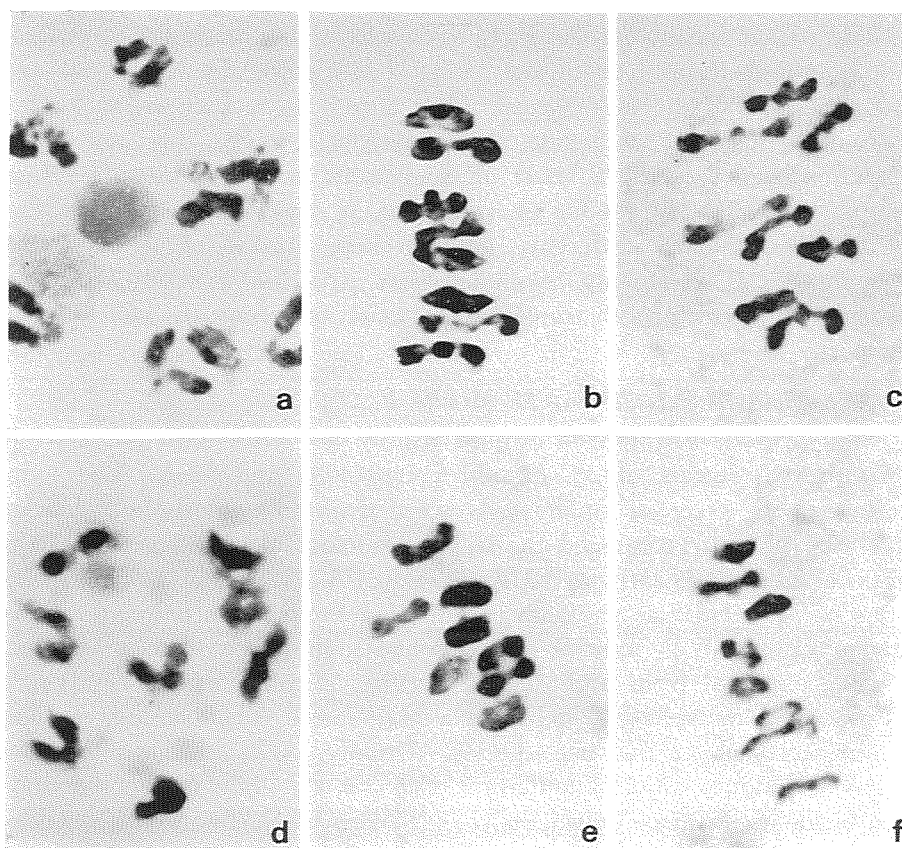


Fig. 3. Different stages in meiosis of the F_1 hybrids examined. $\times 2100$.

- a. *M. officinalis* P.I. 178985 \times *M. hirsuta* 58-4 F_1 . Diakinesis with 8II.
- b. *M. officinalis* P.I. 178985 \times *M. hirsuta* P.I. 314096 F_1 . Metaphase 1 with 8II.
- c. *M. officinalis* P.I. 178985 \times *M. polonica* 3446 F_1 . Metaphase 1 with 8II.
- d. *M. polonica* C 30 \times *M. suaveolens* Redfield Yellow F_1 . Diakinesis with 8II.
- e. The same F_1 as d. Metaphase 1 with 8II.
- f. *M. hirsuta* 58-4 \times *M. suaveolens* Bdn 60-59 F_1 . Metaphase 1 with 8II.

stainable pollen grains. As well as the hybrids between *M. officinalis* and *M. hirsuta*, the course of microsporogenesis in the hybrids, *M. officinalis* P.I. 178985 × *M. polonica* 3446, was regular (Fig. 3 c and Table 4).

f. *M. polonica* × *M. suaveolens* F₁

TABLE 4. Chromosome configurations at diakinesis and metaphase 1 and their distribution in later stages of meiosis in the interspecific F₁ hybrids, showing meiotic chromosome behaviors were regular

cross	no. of cross combinations examined	stage	frequency of PMCs with				total
			8II	7II+2I	normal	abnormal	
<i>M. officinalis</i> × <i>M. hirsuta</i>	2	diakinesis	204	21			225
		metaphase 1	183	23			206
		anaphase 1			120	8	128
		anaphase 2			137	1	138
<i>M. officinalis</i> × <i>M. polonica</i>	1	diakinesis	66	4			70
		metaphase 1	83	19			101
		anaphase 1			56		56
		anaphase 2			41	1	42
<i>M. polonica</i> × <i>M. suaveolens</i>	1	diakinesis	70	1			72
		metaphase 1	84	90			103
		anaphase 1			53	2	55
		anaphase 2			19	1	20
<i>M. hirsuta</i> × <i>M. suaveolens</i>	1	diakinesis	93	10			103
		metaphase 1	97	11			108
		anaphase 1			64		64
		anaphase 2			92	9	101
<i>M. hirsuta</i> × <i>M. polonica</i>	1	diakinesis	41	2			43
		metaphase 1	97	12			109
		anaphase 1			54	5	59
		anaphase 2			42	3	45
<i>M. taurica</i> × <i>M. wolgica</i>	1	diakinesis	125	3			128
		metaphase 1	83	22			105
		anaphase 1			89		89
		anaphase 2			70	2	72
<i>M. dentata</i> × <i>M. taurica</i>	3	diakinesis	198	22			220
		metaphase 1	139	52			191
		anaphase 1			201	19	220
		anaphase 2			107	13	120

In the reciprocal crossings between *M. polonica* and *M. suaveolens*, two strains of *M. polonica* C 30 and 3446 and three strains of *M. suaveolens*, Redfield Yellow, 18-4, and Annua were used. Mature hybrid seeds were obtained at low frequency from only the crosses, *M. polonica* × *M. suaveolens*. Although the hybrid seedlings showed no chlorophyll deficiency at all, they were very weak and several of them died before flowering. Consequently, two F₁ plants from the cross of *M. polonica* C 30 × *M. suaveolens* Redfield Yellow were able to live to maturity. In comparison with the maternal parent, the hybrid plants produced relatively dense racemes.

The pollen of the hybrids were highly sterile (ca. about 20.4% of stainable pollen grains). In the F₁ hybrids, eight bivalents occurred constantly at diakinesis and metaphase 1 (Fig. 3 d, e) and abnormal disjunctions were present in a very low frequency at both anaphase 1 and anaphase 2 (Table 4). Therefore, chromosomal behaviors of meiosis in the hybrids were normal. The pollen sterility in the hybrids, *M. polonica* C 30 × *M. suaveolens* Redfield Yellow, was apparently caused not by chromosome aberrations but by genic factors of one of the parental strains, because of very high pollen sterility of *M. polonica* with the regularity of chromosome behaviors in the course of meiosis, as mentioned previously.

g. *M. hirsuta* × *M. suaveolens* F₁

Three hybrid seedlings obtained at low frequency from the only cross, *M. hirsuta* 58-4 × *M. suaveolens* Bdn 60-59, though crossings were made reciprocally by using several strains of the two species. These seedlings showed heavy chlorophyll deficiency and two of them died soon. But the remaining one had leaflets with sectors of normal green tissue and fortunately a normal green branch arose on the plant. The F₁ hybrid became vigorous and came to flowering. Thus, the hybrid permitted the following observations of meiotic chromosome behaviors.

The percentage of stainable pollen grains of the hybrid was very high, being about 87.6%. At diakinesis and metaphase 1, the chromosome associations such as 8_{II} were regularly present (Fig. 3 f and Table 4). Normal disjunctions of the chromosomes occurred at anaphase 1 and anaphase 2 in almost spore nuclei examined. These observations indicate that the course of meiosis in the F₁ hybrid was normal.

h. The F₁ hybrids among three species, *M. alba*, *M. hirsuta*, and *M. polonica*

Cytological relationship among three species, *M. alba*, *M. hirsuta*, and *M. polonica*, has been investigated by using several strains of each species.

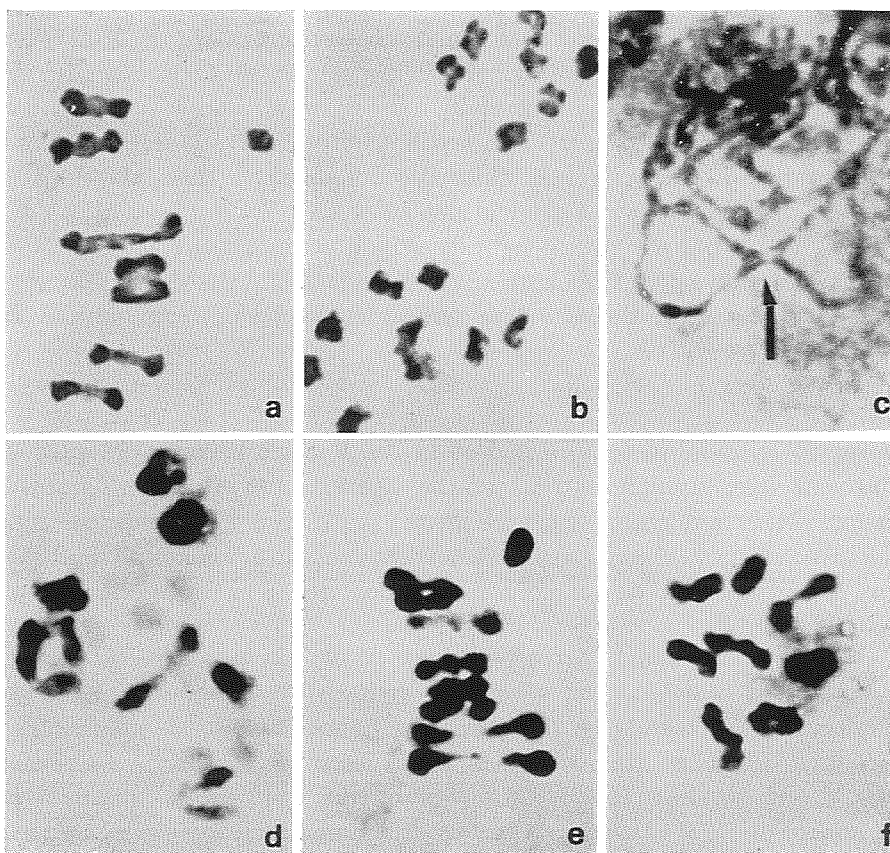


Fig. 4. Different stages in meiosis of the F₁ hybrids examined.

- a. *M. alba* Arctic \times *M. hirsuta* P.I. 314096 F₁. Metaphase 1 showing 1III+6II+1I. $\times 2100$.
- b. *M. alba* Bdn 647 \times *M. hirsuta* P.I. 314096 F₁. Anaphase 1 showing 7-9 disjunction. $\times 2100$.
- c. *M. polonica* C 30 \times *M. alba* Arctic F₁. Pachytene showing a cross-shaped configuration. The breakage point is indicated by arrow. $\times 2450$.
- d. The same F₁ as c. Diakinesis showing 6II plus a ring of 4 chromosomes configuration. $\times 2100$.
- e. The same F₁ as c. Metaphase 1 showing 1III+6II+1I configuration. $\times 2100$.
- f. *M. hirsuta* 58-4 \times *M. polonica* 3446 F₁. Metaphase 1 showing 8II configuration. $\times 2100$.

Cross pollinations were made in order to ascertain cytological relationship among them from observations of meiotic behaviors in the hybrids within several strains used for this study. In reciprocal crossings between *M. alba* and *M. hirsuta*, a great number of F_1 hybrids were readily obtained using several strains of the species. The seedlings from the crossing, *M. alba* \times *M. hirsuta*, showed much less chlorophyll deficiency than those of the reciprocal. Reciprocal cross pollinations between *M. alba* and *M. polonica* also gave many normal green hybrids which were almost more vigorous than the parental species, though the seedlings from the crosses in which *M. alba* was used for the pistillate parent showed slight chlorophyll deficiency in young leaves.

All of these interspecific F_1 hybrids had partial pollen sterility as shown in Table 1. From cytological observations of two cross combinations of F_1 hybrids, meiotic behaviors in the hybrids between *M. alba* and *M. hirsuta* were similar to those of the hybrids between *M. alba* and *M. polonica*. Aberrant chromosome behaviors were observed during the course of meiosis, namely, the quadrivalents were present at diakinesis and metaphase 1, and cross-shaped configurations occurred at pachytene (Fig. 4 a, b, c, d and Table 2).

On the other hand, crossings were made reciprocally between *M. hirsuta* and *M. polonica*, but several F_1 hybrids were obtained from only the crosses, *M. hirsuta* \times *M. polonica*. And these F_1 hybrids showed slight chlorophyll deficiency but reached to the flowering vigorously. As reported by KITA and NIIZEKI (1967), the pollen of the hybrids was highly fertile with about 96.4% of stainable pollen grains, and the chromosome behaviors of meiosis were normal (Fig. 4 f and Table 4).

The observations mentioned above indicate that there are chromosomal differences by one reciprocal translocation both between *M. alba* and *M. hirsuta* and between *M. alba* and *M. polonica*, explaining partial pollen sterility. Moreover, these chromosomal changes are of the same origin, as discussed later, since the course of meiosis in the hybrid, *M. hirsuta* \times *M. polonica*, which had high pollen fertility, was regular.

i. *M. taurica* \times *M. alba* F_1

Generally, the F_1 hybrids between *M. taurica* and *M. alba* showed distinct chlorophyll deficiency and none survived to the flowering stage. In this experiments, unexpectedly, F_1 hybrids from crossings by using two strains of *M. alba*, Cumino and Denta, derived from the crosses of (*M. alba* \times *M. dentata*) \times *M. alba*, as pollen parents, showed somewhat more green color than those from the use of the other strains of *M. alba* and

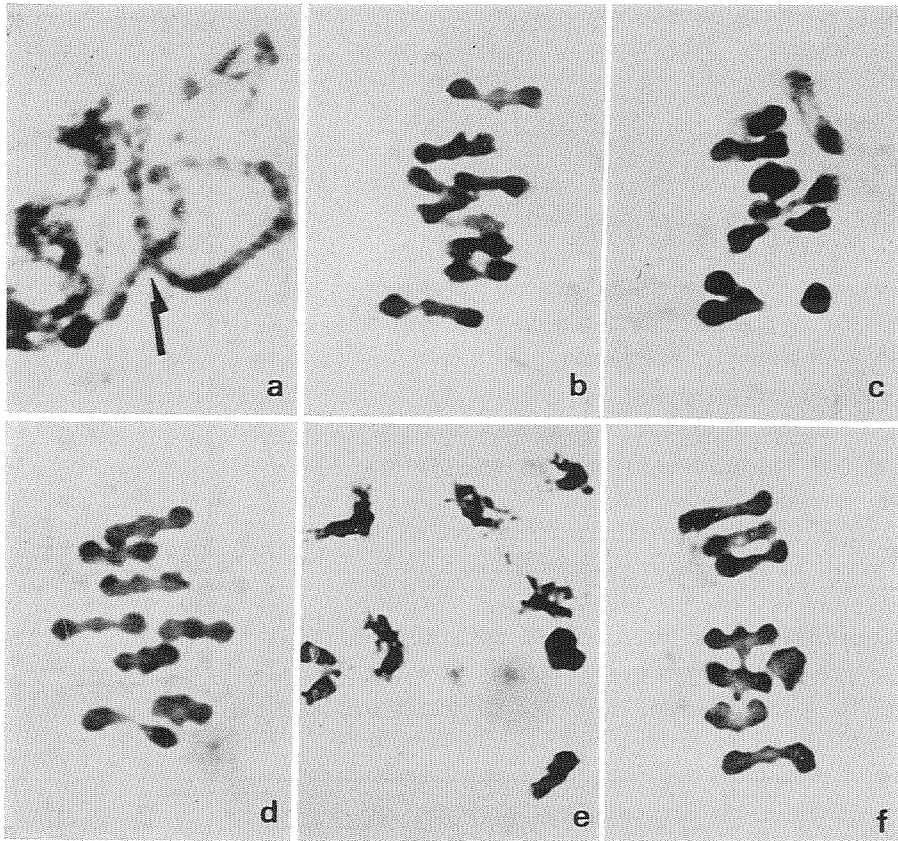


Fig. 5. Different stages in meiosis of the F_1 hybrids examined.

- a. *M. taurica* 70 \times *M. alba* Cumino F_1 . Pachytene with a cross-shaped configuration. The breakage point is indicated by arrow. $\times 2800$.
- b. The same as a. Metaphase 1 with $1_{IV}+6_{II}$ which show alternate disjunction. $\times 2100$.
- c. The same F_1 as a. Metaphase 1 with $1_{III}+6_{II}+1_I$. $\times 2100$.
- d. *M. taurica* 70 \times *M. wolgica* 58-254 F_1 . Metaphase 1 with 8_{II} . $\times 2100$.
- e. *M. dentata* Bdn 58-190 \times *M. taurica* 70 F_1 . Diakinesis with 8_{II} . $\times 2100$.
- f. The same F_1 as e. Metaphase 1 with 8_{II} . $\times 2100$.

however, a great number of hybrid seedlings were more vigorous than those from the reciprocal. When the plants reached the flowering stage, the color of the older leaves was approximately normal green.

The pollen of F_1 hybrids were partially sterile, having about 25.5% of aborted pollen grains. In spite of low pollen fertility, chromosomal behaviors of meiosis in the F_1 hybrids were regular. The configurations

such as 8_{II} at diakinesis and metaphase 1, and normal disjunction at anaphase 1 and anaphase 2 were present in almost spore nuclei (Fig. 5 d and Table 4).

k. *M. dentata* × *M. taurica* F_1

The crossings between *M. dentata* and *M. taurica*, by using three strains of *M. taurica*, 70, 134-1, and P.I. 314094, as pistillate parents, and two strains of *M. dentata*, Bdn 58-190 and P 783-2, as pollen parents, gave only much aborted seeds except for plump seeds resulting from accidental self-pollination of the maternal parent. From the reciprocal crosses, *M. dentata* × *M. taurica*, by using the same strains of two species, several hybrid seedlings were obtained and showed distinct chlorophyll deficiency. But the plants gradually became vigorous and produced pale yellow flowers showing that they were F_1 hybrids since the maternal parent was yellow-flowered.

Although the hybrids had a high percentage of aborted pollen grains (Table 1), the configuration with eight bivalents (Fig. 5 e, f) occurred regularly at diakinesis and metaphase 1 in all cross combinations examined, as well as the hybrid of *M. taurica* × *M. wolgica*. Occasionally, seven bivalents plus two univalents ($7_{II} + 2_I$) were present. Abnormalities such as lagging chromosomes at anaphase 1 and anaphase 2 seldom occurred (Table 4). Hence, it is reasonably pointed out that no structural differences in chromosomes exist between *M. dentata* and *M. taurica*.

Discussion

In regard to cytological studies of the interspecific hybrids in *Eumelilotus* of the genus *Melilotus*, several papers have been reported by the other workers. WEBSTER (1950, 1955) examined meiosis of three interspecific F_1 hybrids, *M. polonica* × *M. svaueolens*, *M. alba* × *M. polonica* and *M. officinalis* × *M. alba*, and pointed out that meiosis in these F_1 hybrids appeared to be as normal as those of the parental species. In the hybrid (*M. alba* × *M. dentata*) × *M. alba*, BRINGHURST (1951) observed cross-shaped configurations at pachytene indicating reciprocal translocation. SHASTRY *et al.* (1960) reexamined the hybrid of *M. officinalis* × *M. alba* reared by embryo culture and arrived at the same result as WEBSTER's. On the other hand, it was revealed that two F_1 hybrids, *M. polonica* × *M. alba* (JARANOWSKI 1961) and *M. alba* × *M. hirsuta* (KITA 1962), were heterozygous for a reciprocal translocation. Accordingly, JARANOWSKI refuted WEBSTER's conclusion (1950) that the chromosome behaviors of the hybrid,

M. alba × *M. polonica*, were regular.

In this report, it became clear that the five F_1 hybrids between *M. alba* and each of the following species, *M. hirsuta*, *M. officinalis*, *M. polonica*, *M. suaveolens* and *M. taurica*, were heterozygous for a reciprocal translocation. Moreover, as far as examined, there is no evidence that intraspecific differences in chromosomal changes exist in any species. This might be also clearly pointed out from the observations that the intraspecific F_1 hybrids in several species had high pollen fertility. It is important to know whether chromosomal changes by one reciprocal translocation between *M. alba* and the other four species are of the same origin or not. The courses of meiosis in the five hybrids, *M. hirsuta* × *M. polonica*, *M. hirsuta* × *M. suaveolens*, *M. officinalis* × *M. hirsuta*, *M. officinalis* × *M. polonica* and *M. polonica* × *M. suaveolens*, were normal. Then, it is reasonably estimated that the course of meiosis in the F_1 hybrid between *M. officinalis* and *M. suaveolens* is normal. These results suggest that the interchange of chromosomes among two pairs of nonhomologous chromosomes had taken place in either *M. alba* or *M. hirsuta*, *M. officinalis*, *M. polonica* and *M. suaveolens* in the process of speciation. In the former case, the reciprocal translocation found in the four interspecific F_1 hybrids are obviously the same origin. In the latter case, however, farther evidences are required to reveal whether the breakage points of chromosomes in the four species are same or not. Now, if the breakage points differ from each other, these four species differ with respect to, what is called, interstitial translocation which do not involve chromosome ends. Because of the way in which chromosomes pair at meiosis, such differences would not be expected to give rise to multivalent rings or chains, but only to the frequent appearance of unpaired chromosomes and/or normal bivalents (STEBBINS 1970). It so, the hybrids within these four species would be partially sterile, since half of the resulting gametes would be deficient for one and duplicated for the other of the interchanged segments. The parental type (fertile) and translocation heterozygote (semisterile) would be expected to appear in the F_2 generation in a 1:1 ratio. The pollen of all most possible F_1 hybrids within these four species was highly fertile, excepted for the hybrid of *M. polonica* C 30 × *M. suaveolens* Redfield Yellow showed high pollen sterility because *M. polonica* C 30 was extremely highly pollen sterile. This indicates that the breakage points do not differ from each other and these interchanges of chromosomes observed in the interspecific F_1 hybrids between *M. alba* and each of four species, *M. hirsuta*, *M. officinalis*, *M. polonica* and *M. suaveolens*, are of

the same origin.

On the other hand, the courses of meiosis in two hybrids, *M. taurica* × *M. wolgica* and *M. dentata* × *M. taurica*, were normal. It is apparent that among these three species there is no gross exchanges of chromosomes such as a reciprocal translocation found in the hybrid between *M. taurica* and *M. alba*. Therefore, the hybrids between *M. alba* and each of the two species, *M. dentata* and *M. wolgica*, which are not obtained yet, seem to be also heterozygous for a reciprocal translocation, agreeing with the result reported by BRINGHURST (1951). There are some difficulties to explain exactly whether three species, *M. dentata*, *M. taurica* and *M. wolgica*, differ from each other with respect to interstitial translocation, since the F₁ hybrids, *M. dentata* × *M. taurica* and *M. taurica* × *M. wolgica*, had high and partial sterility, respectively. However, it is likely to be concluded that structural differences by one reciprocal translocation between *M. alba* and each of the three species, *M. dentata*, *M. taurica* and *M. wolgica* are of the same origin, because the most of F₂ plants of *M. taurica* × *M. wolgica* were highly fertile and all of F₂ plants of *M. dentata* × *M. taurica*, though the number of F₂ plants were not sufficient due to poor seed setting and chlorophyll deficiency, had high pollen sterility. High pollen sterility of *M. dentata* × *M. taurica* F₁ may be caused by genic disharmony rather than chromosomal aberrances.

Cytological relationships among species of *Eumelilotus* obtained in this study are shown in Fig. 6. No hybrid seedling between *M. altissima* and

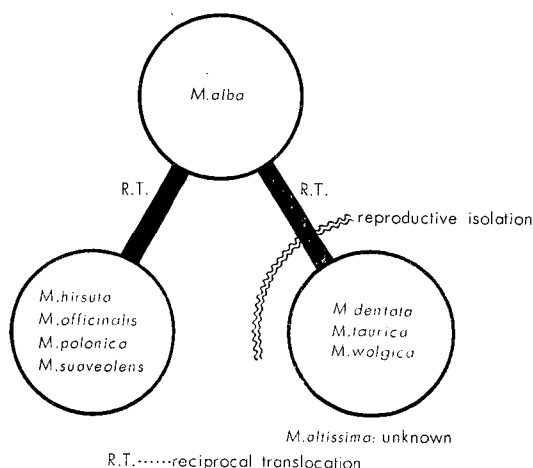


Fig. 6. Cytological relationships among species of the subgenus *Eumelilotus*.

any other species of *Eumelilotus* is able to survive to flowering stage due to heavy chlorophyll deficiency. Therefore, cytological relationships between *M. altissima* and the other species are not revealed at all. Moreover, it is not known whether chromosomal differences between *M. alba* and each of four species, *M. hirsuta*, *M. officinalis*, *M. polonica* and *M. suaveolens*, differ from those between *M. alba* and each of three species, *M. dentata*, *M. taurica*, and *M. wolgica*.

Interspecific differences in chromosome structure are common in many plant genera such as *Clarkia*, *Crepis*, *Elymus*, *Galeopsis*, *Gossypium*, and *Nicotiana*. KYHOS (1965) reported in *Chaenactis* (Compositae) that the two species having $n=5$, *C. fremontii* and *C. stevioides* have evolved from an ancestor similar to *C. glabriuscula* having $n=6$ by a process which included a translocation followed by the loss of a centromere, the translocation being different from each species, as in similar ones found in *Crepis* and so on. In *Clarkia* (Onagraceae), there are several pairs of species which differ from each other with respect to several translocations but the morphological differences among the species are trivial or virtually undetectable (LEWIS 1966). He postulated that in *Clarkia* and perhaps other genera saltational speciation occurs through the simultaneous occurrence of numerous translocations. Also, differentiated sex chromosomes are associated with successive translocations in *Rumex* (B. W. SMITH 1964).

Based on the occurrence of the chromosome aberrations throughout the interspecific hybrids examined by the authors, it is considered that the structural differentiation of chromosomes such as reciprocal translocation plays a significant role during the process of speciation of *Eumelilotus*. It is obviously important to make clear the effects of chromosomal differences such as translocation in speciation. The interspecific hybrids, in the most of cases, show different F_1 weakness, hybrid sterility, and hybrid breakdown, in the advanced generations, which play a role of isolation of species. Farther investigations are interested in making a whole picture of interspecific relationships of *Eumelilotus* from cytological and genic view points.

Summary

Cross pollinations were made among eight species belonging to the subgenus *Eumelilotus* of the genus *Melilotus*. The interspecific F_1 hybrids were examined in order to make clear the cytological relationships among these species systematically.

The results obtained are summarized as follows:

1. *M. officinalis* P.I. 178985 was crossed readily not only with *M. alba* but also with *M. hirsuta* and *M. polonica* and gave vigorous F₁ hybrids. Crossability of the strain of *M. officinalis* is interested in a convenient bridge for the interchange of genetic material among these four species.

2. In the F₁ hybrids, *M. alba* × *M. suaveolens*, *M. officinalis* × *M. alba*, *M. alba* × *M. hirsuta* and the reciprocal, *M. alba* × *M. polonica* and the reciprocal and *M. taurica* × *M. alba*, a cross-shaped configuration at pachytene and a ring or chain of four chromosomes at diakinesis and metaphase 1 were observed, which indicate that the F₁ hybrids are heterozygous for a reciprocal translocation.

3. In the F₁ hybrids, *M. officinalis* × *M. hirsuta*, *M. officinalis* × *M. polonica*, *M. polonica* × *M. suaveolens*, *M. hirsuta* × *M. suaveolens*, *M. hirsuta* × *M. polonica*, *M. dentata* × *M. taurica* and *M. taurica* × *M. wolgica*, the courses of meiosis were regular.

4. As far as examined, there was no evidence that intraspecific differences in chromosomes exist in any species.

5. Consequently it is considered that the F₁ hybrids between *M. alba* and each of two species, *M. dentata* and *M. wolgica*, though the hybrids were not examined, are heterozygous for a reciprocal translocation.

6. Cytological relationships obtained in this studies are as summarized in the figure 6.

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