Biosystematic comparison of species in the genus Agropyron Gaertn. with particular emphasis on serological studies
by Gordon Clifton Creel

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Genetics
Montana State University
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Abstract:
Forty-five strains of 41 species of the genu Aqropyron were investigated to determine their serological relationships. A specially designed test known as the Four-way test was employed for these determinations. Each strain of the 41 species was compared with all others tested and a difference index determined. A difference index of less than 0.12 indicated a close serological affinity. In some cases different species have a difference index of zero. These were given additional test, all of which substantiated the accuracy of the Four-way test.

This investigation clearly shows that, on a serological basis, some of the 4 sections as proposed by Holmberg (1926) are unnatural. The crested wheatgrass complex was distinct from other species tested. An aberrant group composed of A. pycnanthum, A. tsukushiense, A. mayebaranum, and A. scabriglume was found to be distantly related to the other Agropyrons, especially the crested wheatgrass complex, Of all species tested A. pycnanthum was the most aberrant serologically.

The hybrid origin of A. sibiricum was tested and the resulting data rather strongly argue against its origin from a cross of A. repens and A. cristatiforme.

The recognition of A. cristatiforme Sarkar as a distinct species is substantiated. A detailed morphological study of the spike characteristics of the crested wheat grass complex was made. The glume, lemma, and palea were found to be smallest in the diploid, largest in the hexaploid, and intermediate in tetraploid species.

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of

DOCTOR OF PHILOSOPHY

in

Genetics

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Bozeman, Montana

August, 1964

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ABSTRACT

Forty-five strains of 41 species of the genus Agropyron were investigated to determine their serological relationships. A specially designed test known as the Four-way test was employed for these determinations. Each strain of the 41 species was compared with all others tested and a difference index determined. A difference index of less than 0.12 indicated a close serological affinity. In some cases different species have a difference index of zero. These were given additional test, all of which substantiated the accuracy of the Four-way test.

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The hybrid origin of A. sibiricum was tested and the resulting data rather strongly argue against its origin from a cross of A. repens and A. cristatiforme.

The recognition of A. cristatiforme Sarkar as a distinct species is substantiated. A detailed morphological study of the spike characteristics of the crested wheat grass complex was made. The glume, lemma, and palea were found to be smallest in the diploid, largest in the hexaploid, and intermediate in tetraploid species.

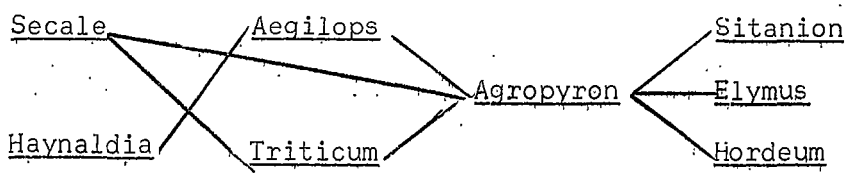
INTRODUCTION

The genus Agropyron of the Gramineae is cosmopolitan in distribution with about 100 of the 150 recognized species indigenous to Eurasia, about 30 species to North America, and the remainder to South America and the Australian region. The Agropyrons are of considerable economic importance, some being excellent forage grasses and others serious pests.

With the coming of modern transportation and an ever-increasing interest in forage crops, the more desirable Agropyron species were introduced and tested in areas other than their native habitat. Breeders in North America began to introduce foreign Agropyron grasses as early as 1898. A number of these have become well established in the United States and are now important crops. The crested wheatgrasses, native to Eurasia, are one of the important groups which have been introduced.

As knowledge of cytogenetics increased and investigators began to make interspecific hybrids, it became apparent that the wheatgrasses could contribute desirable genes, through introgressive hybridization, to important crop species such as wheat.

The genus Agropyron is the largest within the tribe Triticeae (Hordeae) of the Gramineae. In addition to the genus Agropyron, 9 other genera are classified as belonging to the tribe Triticeae. Clausen, Keck, and Hiesey (1945) stated that eight genera belong to the same comparium which Godley (1951) diagrammed as follows:



Stebbins (1961) recommended adding two other genera which are generally included in this tribe. These are the genera Hystrix and Heteranthelium. According to Godley (1951) and Stebbins (1961) Agropyron forms the important connecting link between the genera Secale, Aegilops, Haynaldia, Triticum, and Heteranthelium on the one hand, and Sitanion, Elymus, Hordeum, and Hystrix on the other.

Agropyron has been regarded as an artificial genus by Nevski (1934), Parodi (cited by Conner, 1954) and Stebbins and Walters (1949). Stebbins and Walters concluded that there was not enough evidence to determine accurately the boundaries of the genus. They were of the opinion that the established species names should be retained until more adequate cytogenetic and morphological information would permit the development of a natural system.

It is generally agreed that much confusion exists regarding the status of the genus Agropyron and regarding many species within the genus. A number of workers are studying the cytogenetics and the breeding behavior of the members of the genus, while others are investigating the morphology and anatomy.

A research program at Montana State College has been concerned with the status of the Agropyron species. Schulz-Schaeffer and Jurasits (1962) and Schulz-Schaeffer, et al. (1963b) determined the

karyotypes of 33 species of the genus. Schulz-Schaeffer, et al. (1963a) studied the nature of the polyploidy in tetraploid and hexaploid Agropyron species of the crested wheatgrasses. Lorenz and Schulz-Schaeffer (1964) used two-dimensional paper chromatography to study the flavonoid compounds of 23 strains of 14 Agropyron species and found a definite parallel with morphological classification. The present study is concerned with a comparison of 45 strains of 41 Agropyron species by serodiagnostic methods.

REVIEW OF THE LITERATURE

Nuttall laid the ground work for the serological approach when in 1901 he published on the phylogenetic relationships of some animals, using a "biological test" of blood components. Many workers adopted and modified his method and extended it to include many species of both plants and animals.

That same year Kowarski (1901) found that "heat-resistant albumose of wheat induces in rabbits the production of precipitins which react strongly with wheat albumin extracts, and weakly or not at all with the albumoses of rye, barley, oat and peas, although the reaction with pea extract was stronger than that with oat extract." Ballner and Burow (1910) showed by complement fixation that rye is serologically more closely related to wheat than to barley and oats. He also found that rice and maize were still more distantly related to rye.

Rives (1923a,b) found, while working with grapes, that "failure to graft successfully was accompanied by a marked serological difference, while varieties which grafted successfully were similar by the test." Green (1926), working with Citrus, Rosaceae, and Solanaceae came to the same conclusions as Rives.

The period 1920-30 was one of much serological activity and some began to believe that this technique would provide an almost completely objective approach to systematics. At Königsberg, Germany, there emerged a very prominent group of phytoserologists who patterned their methods after Gohlke's (1913). Later Mez was

the leading investigator of this school. Mez and Ziegenspeck (1926) published their "Serodiagnostiche Stammbaum" as the culminating work of the Königsberg school. This work was greatly criticized by the Berlin school of serologists, primarily Gilg and Schurhoff (1927), who stated that "the serodiagnostic method is, for investigation of plant relationships, completely useless."

Barner and Helwig (1927) followed the procedure of serology which Mez used, but later drastically altered it. Working with Geraniales, Sapindales, Rhamnales, Malvales, Verticillatae, Ranales, and Primulales, they extracted the proteins (antigens) from powdered seeds by using physiological saline or 0.1 sodium hydroxide. They concluded from their work that "Serodiagnostic methods are ....., not suitable for studies in phylogeny of plants, but are suitable for identification of specific plant proteins or of individual plant species and even for demonstration of relationships of plants within a family."

Animal serologists led the field of biochemical systematics from the late twenties until today. In 1928 Saski published on serological relationships between wild and domestic ducks as determined by the precipitin reaction. He found that he could distinguish the Japanese duck, Anas domestica erecta, from the Chinese goose, Anser cygnoides, but could not distinguish the Japanese duck from the Muscovy duck, Cairina moschata.

Martin and Cotner (1934) made a serological study of moth proteins and attributed special importance to their phylogenetic

significance. They used the classical precipitin test made popular by the Rutgers school of serologists, one of whom was Makino (1934), who worked with several species of mollusks. By means of several cross tests with the precipitin, complement fixation, and anaphylactic reactions, he determined their serological relationships. Levit, et al. (1936) had so perfected the serological method that the complement fixation test could be used to detect the presence of the Y chromosome in males and attached-X females of Drosophila. Cumley (1940), commenting on the work of Levit, et al., said "this suggests the possibility of applying immunological techniques to the study of the expression of individual chromosomes, or even genes."

Levine and Moody (1939) determined serological relationships of a number of rodents and found, with a single exception, agreement with taxonomy based on morphology. They found that the North American porcupine, Erethizon dorsatum (L.), and woodchuck, Marmota Monax (L.), are more closely related than previously thought and that the pocket gopher (Geomyidae), even though placed in the same superfamily as the woodchuck, was not as closely related to it as the porcupine, which is in a distant superfamily.

Cumley (1940) compared the serological and taxonomic relationships of Drosophila caribbea, D. melanogaster, D. mulleri, and D. virilis. He found, by both techniques, that D. melanogaster and D. caribbea were closely related, and likewise D. mulleri and D. virilis.

Cumley, et al. (1943) working with several species of doves, their hybrids, and backcrosses concluded that "the species specific antigens of the serum of these different species are controlled by genes. The inference may well be drawn that all the antigens of the serum of animals, except those antigens which may be metabolic or other products being transported by the serum are gene-determined. Thus a genetic basis of classification is given the systematist who uses serology in his work."

Wilhelmi (1944) made a serological comparison of the Mollusca with other invertebrate phyla and found them most closely related to the Annelida. Leone (1947), using serological methods, was able to determine the relationships of 5 families of Orthoptera. Bacci and Oddo (1948) worked out the relationships of several species of mollusks using serological procedure and their conclusions agreed with the findings of classical taxonomy.

It had long been supposed that the Lagomorpha were more closely related to the Rodentia than to other groups. In 1949 Moody, Cochran, and Drugg performed serological tests which indicated that the lagomorphs are more closely related to the artiodactyls than other groups tested. This work confirmed the separation of lagomorphs and rodents into distinct orders and showed that these orders are not closely related.

Cather (1955) applied serological methods to determine the relationship of some gastropods collected in Dallas County, Texas. The results of his research support the theory that Pulmonata was

derived from the Monotocardia. He also concluded that Limax has apparently undergone considerable protein change since it originated.

The relationship of the different groups of poisonous snakes has been a subject of much concern to herpetologists and evolutionists for a long time. Kuwajima (1953) worked with the venoms and blood sera of the Formosan poisonous snakes as antigens and demonstrated differences and similarities.

Leone (1950a) made serological comparisons of several species and families of European Brachyura and found that his results confirmed the existing classification based upon morphological characters. He was also able to show that sera of the same species of Brachyura collected at widely separated geographical localities showed no difference in their serological activities. In another paper on Crustacea, Leone (1950b) concluded that:

1. Sera of species within a genus react more strongly with an antiserum made against one of them than with any other antiserum.

2. Sera of different species within the same genus may show different degrees of relationship to an antiserum made against the serum of one member of that genus.

3. Sera of representatives of different genera in the same family show different degrees of relationship.

4. Sera of species within a family react to a greater extent with antisera made against the sera of members of that family than with any other antisera.

He also found from this work that freezing, Seitz filtration, centrifugation, and storage for periods up to 15 years caused no change in the serological activity of the hemocyanins.

Leone and Pryor (1954) made a serological study of the 4 Mollusca classes, Amphineura, Gastropoda, Cephalopoda, and Pelecypoda, and found that only extremely slight cross-reactions occurred between classes. They concluded that the 4 groups should be retained as distinct classes.

A study of the comparative serology of the carnivores was conceived and undertaken by Leone and Wiens (1956) in which they compared 7 families of fissipeds and one family of pinnipeds. From this work they came to the conclusion that the serological divergence between canoid and feloid carnivores was sufficient to justify making each group a suborder. They also found that classifying the Pinnipedia as a separate suborder was not justified on the basis of their serological data which placed them within the Canoidea.

Mainardi (1959) undertook a serological study of the relationship of several gallinaceous birds. He found that the ring-neck pheasant (Phasianus colchicus L.), guinea (Numida meleagris), and turkey (Meleagris gallopavo L.) have a close immunological affinity in spite of the fact they belong to three different families (Phasianidea, Numidinae, Melleagridae). He also found that the domestic chicken (Gallus gallus) and Japanese quail (Coturnix) which belong to the same family as the ring-neck pheasant are rather remote serologically from one another and from all other species studied.

Moody and Doniger (1956) made a serological comparison of old and new world porcupines, Hystrix and Erethizon dorsatum (L.) respectfully, and found them to be rather remotely related as indicated by their slight serological similarity. Sindermann and Mairs (1960), working with both agar diffusion and turbidometric methods, studied the serological relationship of several fishes. They found that alewives, Alosa pseudoharengus (Wilson), and blueback salmon, Oncorhynchus nerka (Walbaum), have a high degree of serological similarity. Common shad were found to be closer to the alewife-blueback complex than to either menhaden, Brevoortia tyrannus, or herring, Clupea harengus; and menhaden and herring were remote from all others tested and from each other. Herring showed the greatest serological difference.

Although most serologists have worked with animals, a scattered few plant taxonomists have maintained a confidence in the method for their purposes, and in the last 15 years investigations have been made with results apparently as useful as those of the animal systematists.

Baldwin, et al. (1927) made a serological comparison of 29 species of cultivated legumes using precipitin and anaphylaxis reactions. They were interested in correlating serological relationship with cross inoculation. The results of their research showed "that all members of any cross inoculation group are closely related with respect to the protein characteristic of their seeds,

and in the majority of cases all legumes which possess closely related seed protein complexes cross inoculate."

Hyun (1949) made a serodiagnostic investigation of the affinities of different species of the genus Quercus. He extracted proteins with physiological saline from seeds in the first stage of germination and injected them into rabbits to stimulate antibody production. His interpretation of the data was that serological relationships between the 15 species of Quercus coincided with the treatment of systematic botanists. The serology indicated that Q. mangorica and Q. aliena are between Q. serrata and Q. dentata in relationship. Of interest was his discovery that the evergreen Q. phylliraeoides is more closely related to the deciduous Q. variabilis and Q. acutissima than to other evergreen oaks.

Johnson (1954) made a serological comparison of several genera of Magnoliaceae by comparing them to Magnolia. He also compared several species of Magnolia in an effort to establish intrageneric relationships. His intergeneric serological comparisons confirmed the classical taxonomy grouping. He found that Magnolia, Michelia, and Talauma did indeed form a natural group, and that Liriodendron is relatively distant. He also found that Illicium, which had been removed from the Magnoliaceae by earlier workers, gave no reaction when tested with Magnolia antiserum. McLaughlin (1933) had placed the genus Illicium in the Hamamelidaceae, but Johnson (1954) found that Disanthus, also in the family Hamamelidaceae, gave no serological

reaction with Illicum. This would perhaps support the treatment of some taxonomists of a monotypic family, Illiciaceae. Johnson's investigation at the species level revealed that the Asiatic species Magnolia obovata is more closely related to the American species M. tripetala than M. tripetala is to two other American species, M. acuminata and M. virginiana. His data indicated that Magnolia portoricensis is farthest removed of any species checked. Serological and morphological evidence supports the proposal that it be placed in a separate subgenus.

Hammond (1955) did some serological work with several genera of Ranunculaceae and as a result of this and a morphological study he produced a new systematic treatment of the genera. Hammond's work with this family led him to the conclusion that the family is serologically close-knit. One of the outstanding results of Hammond's work was his placement of Hydrastis into the Ranunculaceae. This action was based on a positive reaction with Aquilegia antiserum.

Gell, et al. (1960) with the application of immunological methods, studied the taxonomy of some species of the genus Solanum. They used the gel-diffusion technique developed by Elek (1948) and Ouchterlony (1948) with some modifications, notably the application of immuno-electrophoresis developed by Grabar and Williams (1953). They found that what appeared as a single line in the gel could often be separated into two or more by use of the immuno-electrophoretic technique. Using absorbed antisera, they were able to divide the

species of Mexican potatoes into 4 well-defined groups. They concluded that "Morphological considerations then, indicate a rough correlation with the serological tests, though some exceptions can be noted. With the exception of the linking series Polydenia and the possibly linking series Bulbocastana, the serological results follow quite closely those obtained from crossability and morphological studies."

Tucker (1963) working with two subspecies of Marchantia polymorpha was able to show a serological difference between them. The procedure which he used was essentially that used by Gell, et al. (1960).

Serological techniques have made a significant contribution in research with hybrids. One of the classical papers on the serological study of animal hybrids was that of Irwin (1951) who worked with Columbidae and studied the serology of the Ring dove, Streptopelia risoria Linnaeus, Pearlneck dove, S. chiensis Scopoli, and their hybrids. He found that the hybrids had the antigens of both parents and some antigens not found in either parent. McGibbon (1944) was able to demonstrate the presence of antigens in hybrid ducks which did not occur in either parent. He found that after injecting this "hybrid antigen" into the parents, both produced antibodies in response, thus demonstrating its uniqueness in the hybrid.

Fox, et al. (1961) found that hybrids of the toads Bufo fowleri Hinckley and B. valliceps Wiegmann had all the components of the parents but no hybrid antigens.

Hybrids from species of several families of plants have also been studied serologically. Zade (1914) published on the serology of some legumes and one part of his work was a serological comparison of Trifolium repens, T. pratense, and T. hybridum. Trifolium hybridum was considered to be of hybrid origin as the epithet indicates resulting from a cross of T. repens and T. pratense. The serological data of this work led Zade to the conclusion that T. hybridum was indeed of hybrid origin and derived from the supposed parents. Chester (1937a,b) wrote "Zade, with precipitin test, showed that Trifolium pratense and T. repens are related, but serologically distinct, their hybrid, T. hybridum reacts so strongly with both as to demonstrate its hybrid nature."

Moritz and vom Berg (1931) working with Vicia leganyi (hybrid) and its supposed parents Lens esculenta and Vicia sativa decided that it was of hybrid origin in spite of the observation that some of the proteins of Lens esculenta were absent in the hybrid. Moritz (1958), working with amphidiploid hybrids, Aegilops ovata X Triticum dicoccoides and Triticum aestivum X Secale cereale, found them to be serologically intermediate to the parents.

Hall (1959) used the immuno-electrophoretic technique descried by Grabar (1955) to study the macromolecular composition of a rye-wheat hybrid and its parents. The macromolecules analysed were extracted from dry seeds, which were ground into a fine powder, with 0.85% sodium chloride. His conclusions were ---1) immuno-electrophoretic identity has been established between some soluble

seed proteins from wheat and rye, 2) all proteins immuno-electrophoretically identified in the wheat could be traced in the rye-wheat, 3) indications have been found that rye-wheat is unable to produce some of the proteins specific for rye, and 4) specific rye-wheat proteins could not be found.

Alston and Turner (1963) wrote "Suppose, for example, that species A contains antigen complement a + b and species B contains b + c. Thus b, represents the common antigenic substances. The hybrid should, therefore, possess a complement a + b + c, and a hybrid antiserum, if absorbed with serum type A and then serum type B, should be completely neutralized. Presumably then, if a residual activity remained in the antiserum after absorption with sera A and B, one of three explanations might hold:

(1) The plant was not a hybrid.
(2) New hybrid-type antigenic substances were present.
(3) Genetic heterozygosity in one or both parents led to individual differences in antigenic complement.

However, if serum of the "hybrid" is completely neutralized by antisera of type A and type B, this result offers strong support for the true hybrid nature of the plant in question."

Troitskii (1932) and Tronickij (1959) indicated that A. sibiricum probably is of hybrid origin resulting from a hybrid between A. repens and A. cristatum (diploid A. cristatiforme). It seems possible that this could be true when the confusion regarding the statues of the genus Agropyron and some of its species is considered.

A conflict concerning the type species of this genus has complicated the work of taxonomists. Jones (1960) wrote that when Gaertner described the genus Agropyron, only two species were included (Novi Comm. Petrop. XIV p. 531, 1770). The first one treated was A. cristatum (L.) Gaertn. which was based on the perennial Bromus cristatus, and the second, A. triticeum, was described as a new species. The description of A. triticeum was accompanied by detailed drawings. Britton and Brown (1913) (cited by Jones, 1960) chose A. cristatum as the type species. Hitchcock (1920), unaware of the work of Britton and Brown, selected A. triticeum Gaertn. as the generic type. He selected this species because Gaertner included detailed drawings with his work in describing the genus.

Nevski (1934) amended the genus Agropyron and chose A. cristatum (L.) Gaertn. as the type species. In this amended work he placed A. triticeum Gaertn. into a new genus, Eremopyrum (Ledeb.) Jaub. and Spach., along with the other annuals. Recently Skalicky and Jirasek (1959) have returned the annual species of Eremopyrum to the genus Agropyron. They also have transferred the species of Agropyron Gaertn. em. Nevski (the crested wheatgrasses) to the genus Kratzmannia Opiz.

Nevski's (1934) generic and subgeneric names for the wheatgrass complex are generally substituted for by the sections of the genus Agropyron published by Holmberg in 1926. The sections are 1) Goulardia (Husnot) Holmberg, 2) Holopyron Holmberg, 3) Agropyron, and 4) Eremopyrum.

MATERIALS AND METHODS

Forty-five strains of 41 Agropyron species were analyzed in this study. Seeds of these species were obtained from research institutions, plant material centers, and botanic gardens. Accession numbers, names of collection locations, and observed chromosome numbers are reported in Table 1. Voucher specimens are located in the Montana State College Herbarium.

All plants used in this study were grown in the Montana State College grass nursery or in a greenhouse. The leaf material used for this study was from adult plants with new vegetative growth or from seedling plants allowed to reach a height of about 15 cm. before being cut. When seedlings were used, many plants were cut and squeezed as a composite lot. Otherwise a single plant was used as representative of the species except in the cases of A. elongatum, where three different plants were used, representing the three ploidy levels (each was treated separately. Two strains of A. desertorum and A. trichophorum were treated.

The leaf material from a species was immediately placed in marked paper bags to eliminate error. The cut leaves were taken to the laboratory where they were washed at least twice. The adhering water was absorbed as completely as possible by blotting to prevent dilution of the extracts. After blotting, the leaves were placed in a metal cylinder which had a one inch inside diameter and was 4 inches long (Figure 1). The leaves were chopped firmly by hand

Table I. Sources of 45 strains of 41 Agropyron species, used as antigens in this study.

Species	Section	MSC Field No.	Source
A. acutum Roem. et Schult.	Hol.	0.5-2	P.I. 202,727 Botanic Gardens, Brussels, Belgium. 2n=30, 34, 35, 36, 37, 42 (Schulz-Schaeffer and Jurasits, 1962). MONT. 58,418.
A. agroelymoides (Hicken) Hunziker	Gou.	115-16	Hunziker 021. Baleares Province, Buenos Aires, Argentina.
A. albicans Scribn, et Smith	Hol.	111-12	Jenkins 4E6, Central Exp. Farms, Canada
A. angustiglume Nevski	Gou.	141-S	Seed received from Botanical Gardens, Moscow, USSR, No. 1254, via Dr. Christian Lehmann, Gatersleben, Germany, March 2, 1962.
A. arizonicum Scribn. et Smith	Gou.	2-8	Received from seed collection of the Institut für Pflanzenbau und Pflanzenzüchtung Göttingen, Germany, in January, 1960, as field Nos. 52-1957 and 165-1958. 2n=28, determined by Jurasits in 1960. MONT. 59,277.
A. brachyphyllum Boiss. et Haussk.	Gou.	3-2	Collected by H.S. Gentry, east base of Kuhe Zard, Charmahal, Iran, November 9, 1955. 2n=42 (Schulz-Schaeffer and Jurasits, 1962), n=21. MONT. 59,278.
A. caespitosum Grossh.	Hol.	4a(61)-1	P. I. 228,276 (Pullman, Wash.) Collected by H.S. Gentry, 25 miles NW of Shar Kard, Charmahal, Iran, on Oct. 3, 1955, 2n=42 (Schulz-Schaeffer and Jurasits, 1962).

Table I. (continued)

Species	Section	MSC Field No.	Source
A. ciliatiflorum Roshev.	Hol.	9-9	P.I. 207,452. Collected by H.S. Gentry at Paghman, Kabul Province, Afghanistan, Sept. to Oct. 1953. 2n=28 (Schulz-Schaeffer and Jurasits, 1962). MONT. 59,280.
A. cristatiforme Sarkar	Agr.	10-3	Seed from Hungary via Gatersleben, Germany. Received through Institut für Pflanzenbau und Pflanzenzüchtung, Göttingen, Germany, in January, 1960, as field Nos. 1-1957 and 126-1958. Received as A. cristatum. Reidentified by Dr. J.R. Schaeffer, J. Ericson, and Gordon Creel. 2n=14, (Creel, Ericson, and Schulz-Schaeffer, 1964).
A. cristatum (L.) Gaertn.	Agr.	80a-2	P. I. 223,323. Collected by E.E. Smith on road from Meshkinshahr to Ahar, Azerbaijan, Iran, on January 18, 1955. originally introduced as A. squarrosum. Reidentified by Dr. J.R. Swallen in 1962 as A. cristatum. 2n=42 (Schulz-Schaeffer and Jurasits, 1962). MONT. 58,420.
A. dasystachyum (Hook.) Scribn.	Hol.	147-S	P. I. 236,663. Received through Dr. Douglas Dewey, Agricultural Research Service, USDA, Logan, Utah. 2n=28.
A. desertorum (Fisch.) Schult.	Agr.	25-9	P. I. 19,538. Presented by Mr. Vasili S. Boydan, through Prof. M. Golenkin, director of Moskow Botanic Gardens, on December 12, 1906. Collected from Valuiki, Samara Government, Russia. 2n=28 (Creel, Ericson, and Schulz-Schaeffer, 1964) n=14. MONT. 59,291.

Table I. (continued)

Species	Section	MSC Field No.	Source
		30-9	Alma-Ata, Russia. 2n=28 (Creel, Ericson, and Schulz-Schaeffer, 1964).
A. donianum Buch.-White	Gou.	32-1	Received from Botanic Gardens, Cambridge, England, during March, 1960. MONT. 59,296.
A. elongatiforme Drob.	Hol.	33-3	P. I. 222,960. Collected by E.E. Smith, 16 miles east of Tabriz, road to Sarab, Azerbaijan, Iran, January 4, 1955. 2n=58 (Schulz-Schaeffer and Jurasits, 1962).
A. elongatum (Host.) Beauv.	Hol.	35a-2	Collected by Augustin Labbe in the vicinity of Saint Germain near Tunis, Tunisia, November, 1947. 2n=14 (Schulz-Schaeffer and Jurasits, 1962) MONT. 59,297.
		37-5	P. I. 119,603. Collected by H. L. Wellman from the ruins in Troy, Turkey, September 18, 1936. Original seed sample labeled A. intermedium (Host.) Beauv. Reidentified by Dr. J. R. Swallen. P-5330. Strain "Green". 2n=42 determined by Jurasits in 1960. MONT. 59,299.
		36-5	P. I. 98,526. Introduced by N. I. Vavilov from the Saratov Institute, Russia, on April 8, 1932. P-2326. Strains "Nebraska 98,526" and "Blue Type". 2n=71. Determined by Ericson in 1964. MONT. 59,298

Table I. (continued)

Species	Section	MSC Field No.	Source
A. fibrosum (Schrenk) Nevski	Gou.	112-5	Seed sent from All Union Institute of Plant Industry, U.S.S.R.. Collection in Lineck region, Russia (33869).
A. hirsutum (Bertol.) Nevski	Ere.	A1-S	P. I. 229,425. From Pullman, Washington. Received as A. orientale. Reidentified as A. hirsutum by Creel and Ericson in 1964. Collected in Iran.
A. imbricatum (M.B.) Roem. et Schult.	Agr.	28-9	Seed from Alpine Gardens Kirovsk, region Murmansk, Russia. Received through Dr. Christian Lehmann, Institut für Kulturpflanzenforschung, Gatersleben, Germany. Received as A. desertorum, reidentified by Creel, Ericson, and Schulz-Schaeffer. 2n=28, 30, 32, 34, 36 (Creel, Ericson, and Schulz-Schaeffer, 1964) MONT. 59,293.
A. intermedium (Host.) Beauv.	Hol.	44-6	Collected in the Kopet-Dag Mountains, Turkmen S.S.R., Russia. Seed received through Botanic Gardens, Turkmen Scientific Academy, Ashkhabad, Russia, via Dr. Christian Lehmann, Institut für Kulturpflanzenforschung, Gatersleben, Germany on May 27, 1960. 2n=42. Determined by Jurasits in 1960. MONT. 59,305.
A. junceum subsp. mediterraneum (L.) S.	Hol.	140-3	France. Origine Boucherdi Rhone i62. 2n=42 (Cauderon, 1958).

Table I. (continued)

Species	Section	MSC Field No.	Source
A. kosanini Nab.	Hol.	46a-2	P. I. 237,636. Collected by R. K. Godfrey near Zara, Sivas, Turkey, January 6, 1953. Separated from P. I. 204,425 (Bromus) and assigned new P. I. number by Regional Plant Introduction Station, Ames, Iowa. 2n=56 (Schulz-Schaeffer and Jurasits, 1962). n=28. MONT. 59,308.
A. latiglume (Scribn. et Smith) Rydb.	Gou.	47-3	P. I. 236,673. Collected by F. J. Herman and B. M. Leese at Sentinal Peak, Coleman, Kananaskes Road, Canada. 1956 2n=28 (Schulz-Schaeffer and Jurasits, 1962). n=14. MONT. 59,309.
A. littorale (Host) Dum.	Hol.	137-5	France. No. 643, Bases Pyrinees, 2n=42 (Cauderon, 1958).
A. mayebaranum var. mayebaranum Honda	Hol.	149-S	From S. Sakamoto (6387). National Institute of Genetics Misima, Sizuoka-ken, Japan. 2n=42, n=21 (Sakamoto, 1958).
A. mongolicum Keng	Agr.	48b-1	Collected during the Asiatic Expedition in the Suiyuan Province, China, during the period Aug. 9 to Sept. 2, 1935. USDA plant intro. 2n=28, 30, 31, 32, 34, 36. (Creel Ericson, and Schulz-Schaeffer, 1964).
A. panormitanum	Gou.	50a-7	P. I. 197,569. Received from Colonial Botanic Gardens, Palermo, Italy, July, 1951. 2n=28 (Schulz-Schaeffer and Jurasits, 1962). n=14. MONT. 58,421.

Table I. (continued)

Species	Section	MSC Field No.	Source
A. pauciflorum (Schwein.) Hitchc.	Gou.	121-4	Received from Dr. Jenkins, 4E47. Central Experimental Farms, Canada.
A. pungens (Pers.) Roem. et Schult.	Hol.	145-S	Seed received from Botanic Gardens, Taschkent, Russia, via Gatersleben, Germany, on Jan. 8, 1959. 2n=42. Determined by Jurasits in 1960.
A. pycnanthum Gren. et Godr.		128-S	France. Rie sur Belon (Finistere). Received via Dr. Christian Lehmann, Gatersleben, Germany.
A. repens (L.) Beauv.	Hol.	146-S	Received from All Union Institute of Plant Industry Leningrad, U.S.S.R. Collected in the region of Cheliabinsk, Russia. 2n=42. Determined by Jurasits in 1960.
A. riparium Scribn. et Smith	Hol.	62a-2	Collected by R. G. Johnson as A. smithii Rydb. near Canyon City, Grant County, Oregon, in area of 12 inch rainfall at elevation of 3000 feet in 1933. P-2415. 2n=42 (Schulz-Schaeffer and Jurasits, 1962). MONT. 59, 318.
A. scabrifolium (Doell) Parodi	Hol.	138-3	Received from Dr. Hunziker. Argentina, Province Cardoba, La Calera Cutl. Castelar. 4-I-1962. Hunz. iiiY025. 2n=42. Determined by Jurasits in 1960.
A. scabriglume (Hack.) Parodi	Hol.	1-5	P. I. 202,147. Collected by Alan A. Beetle, in the Parodi Grass Garden, Facultad Agronomia Buenos Aires, Argentina, from

Table I. (continued)

Species	Section	MSC Field No.	Source
			individual plant No. 16, February 19, 1952. 2n=42 (Schulz-Schaeffer and Jurasits, 1962). MONT. 59,319.
A. semicostatum (Steud.) Nees	Gou.	64-8	P. I. 203,242. Seeds presented by K. Ehara, Department of Agriculture, Kyushu University, Fukuoka, Japan, on October 22, 1952. Introduced from Manchuria. 2n=42. n=21. MONT. 59,320.
A. sibiricum (Willd.) P.B.	Agr.	68-5	Collected in the Kopet-Dag Mountains Turkmen S.S.R., Russia. Seeds received through Botanic Gardens, Turkmen Scientific Academy. 2n=28, n=14 (Creel, Ericson, and Schulz-Schaeffer, 1964). MONT. 59,322.
A. spicatum (Pursh.) Scribn. et Smith	Hol.	75-3	Grass and Legume Standard Accession List. USDA Soil Conservation Service, Pullman, Washington, Feb. 1955. P-6409. 2n=28. Determined by Jurasits in 1960. MONT. 59,327.
A. stipaefolium Czern.	Hol.	122-2	Received from All Union Institute of Plant Industry. Leningrad, U.S.S.R. Collected in Czern Lineck region, near Leningrad, Russia.
A. trachycaulum (Link) Malte.	Gou.	93-4	Collected by the USDA Forest Service near Beebe, Montana, 1933. Variety "Primar". P-2535. 2n=28. MONT. 59,338.

Table I. (continued)

Species	Section	MSC Field No.	Source
A. trichophorum (Link) Richt.	Hol.	57-5	P. I. 106,831. Introduced as A. popovii. Seed received from the Botanic Gardens, Turkmen Scientific Academy, Ashkhabad, Turkmen S.S.R., Russia, October, 1934. 2n=42. Determined by Jurasits in 1960. MONT. 59,315.
		104-3	Seed received from seed collection of the Institut für Pflanzenbau und Pflanzenzüchtung, Göttingen, Germany, January, 1960, as field Nos. 40-1957 and 138-1958. B-80. 2n-42. MONT. 59,344.
A. triticeum Gaertn.	Ere.	125-S	Collected by Dr. W.E. Booth, near Gardiner, Montana, in 1952. 2n=14. Determined by Ericson in 1964.
A. tsukushiense var. transiens (Honda)		148-S	Received from D.S. Sakamoto, National Institute of Genetics, Misima, Sizuoka-ken, Japan. 6380. 2n-42, n=21 (Sakamoto, 1958).

Explanation of Accession Numbers and Abbreviations:

Agr. Section Agropyron

B. Seed collection of the Department of Plant and Soil Science, Montana State College, Bozeman, Montana

Ere. Section Eremopyrum

Gou. Section Goulardia

Gr. Seed collection of the Institute für Kulturpflanzenforschung, Gatersleben, Germany

Hol. Section Holopyron

MONT. Herbarium of Montana State College, Bozeman, Montana, U.S.A. Lanjouro, J. and Stafleu, F. A. Index Herbariorum. Part I. The herbaria of the world. International Bureau for Plant Taxonomy and Nomenclature, Utrecht, 1954

P Seed collection of the USDA, Soil Conservation Service, Pacific Region, Plant Material Center, Washington State University, Pullman, Washington

P.I. Seed collection of the USDA, ARS, New Crops Research Branch, Crops Research Division, Plant Introduction Stations, Western Region, Pullman, Washington and North Central Region, Ames, Iowa

S Indicates that several seedlings were used rather than a single plant

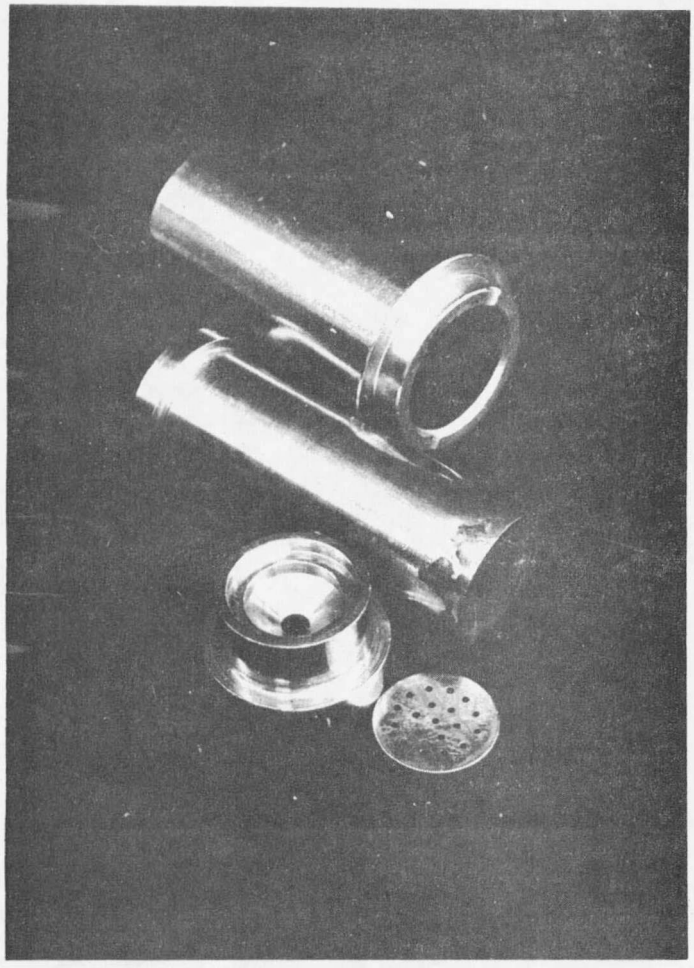


Fig. 1 Cylinder, plunger, and cup used in extracting crude sap.

and then pressed with 5000 pounds of pressure with the aid of a hydraulic jack (Figure 2). Approximately 50 grams of leaf material were squeezed at a time, yielding about 10 ml. of crude extract. The crude extract was placed in sterile vials of 2 drams capacity, about one ml. to a vial, and was used within 2 hours or was immediately frozen at -20° C. until used. The extract was discarded following its use after the second thawing. No preservative nor centrifugation was used, except for the antigen absorption test where it was centrifuged to remove precipitate resulting from reaction of antibodies and antigens.

New Zealand White rabbits were used to produce antibodies in response to injected crude extracts (antigens). A rabbit was injected with antigens from a single species of Agropyron (Table II). Five rabbits were used, each injected with crude extract from a different species. These species were A. cristatiforme (#10-3), section Agropyron; A. triticeum (#125-S), section Eremopyrum; A. dasystachyum (#147-S), section Holopyron; A. panormitanum (#50a-7), section Goulardia; and A. sibiricum (#68-5), section Agropyron. The injections were given in a weekly series beginning with ½ cc. and followed by 1 cc., 2 cc., 4cc. and a final 4cc. Following this series, the antibody level was sufficiently high for use in the gel-diffusion process. Booster injections of 1 cc. were given approximately once a week to keep the antibody level high. All injections were given sub-cutaneously in the lumbar region, except for some booster injections which were given intravenously. The injections were alternated on right and left.

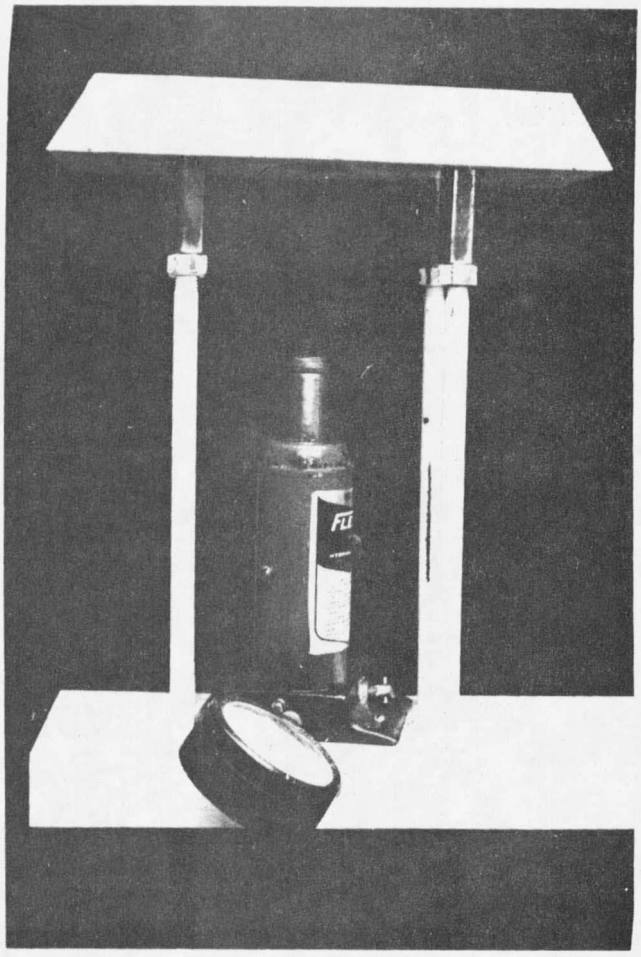


Fig. 2 Apparatus used to apply 5000 pounds of pressure to express crude sap from grass leaves.

Table II. Agropyron species used to induce antibody production in New Zealand rabbits. A species has been selected for each of the four sections of the genus. Species marked with an asterisk were used in the Four-way test.

Species	MSC Field No.	Section
A. cristatiforme*	10-3	Agropyron
A. sibiricum	68-5	Agropyron
A. panormitanum*	50a-7	Goulardia (Husnot) Holmberg
A. triticeum*	125-S	Eremopyrum (Ledeb.) Boiss.
A. dasystachyum*	147-S	Holopyron Holmberg

sides. The rabbits were kept in individual cages. Rabbits of both sexes were used.

Seven days following the last injection, and weekly thereafter as needed, the rabbits were bled from the ear. The ear was shaved and sterilized in the region where the blood was to be taken. The arteries and veins were dilated by application of xylene by cotton swab. After good dilation the lateral ear vein was slit with a razor blade for about $\frac{1}{4}$ inch and the blood collected in a sterile petri dish. About 60 ml. of whole blood were taken at each bleeding, after which the rabbit usually stopped bleeding without assistance.

The blood was allowed to coagulate in the covered petri dish for about 3 hours. Following this the serum was removed by pipette and centrifuged for 20 minutes at 3000 rpm to remove remaining erythrocytes, leucocytes, and platelets. The serum was divided into small aliquots of about 1 ml. each and used within two hours or stored at -20° C. No preservative was added. The serum was discarded following its use after the second thawing. Each container was marked with the date of bleeding and the species name of the plant injected. Serum from each bleeding was treated as a separate lot.

The gel-diffusion plates were prepared as follows: All ingredients but agar were made up into a stock solution by adding 10.3 gr. sodium barbitol, 1.4 ml. concentrated hydrochloric acid, 9 gr. sodium

chloride, and 0.1 gr. sodium azide to distilled water to a volume of 1000 ml. One gram of agar was added to each 100 ml. of stock solution, autoclaved, and 9 ml. quantities dispensed into small (90 mm. diameter) petri dishes. The agar preparation was buffered by the ingredients to a pH of about 7.2. The agar was allowed to harden for several hours before use and was about 1.5 mm. in depth, but varied somewhat depending on the flatness of the petri dish bottom.

After the agar in the petri dishes was set (congealed), wells and a trench were cut in each following a template (Figure 3) for accuracy. The elongate antiserum trench was centrally located on the plate and measured 5 x 76 mm. A row of 5 antigen wells was placed along the two long sides of the antiserum trench. The antigen wells were made with a #3 cork borer (Figure 3), tapered to the inside, and were 8 mm. in diameter, 8 mm. apart, and 7.5 mm. from the antiserum trench. The antiserum trench was made by cutting the agar with an X-acto knife (Figure 3) following the template pattern. The agar plugs were removed by vacuum. The agar plates were allowed to stand, at room temperature, for several hours before inoculation with antigens and antiserum.

Each agar plate was numbered by using a heated probe to engrave the number in the agar, thus eliminating the possibility of error of identification. Each numbered agar plate had a correspondingly numbered record card with the details of the experiment recorded.

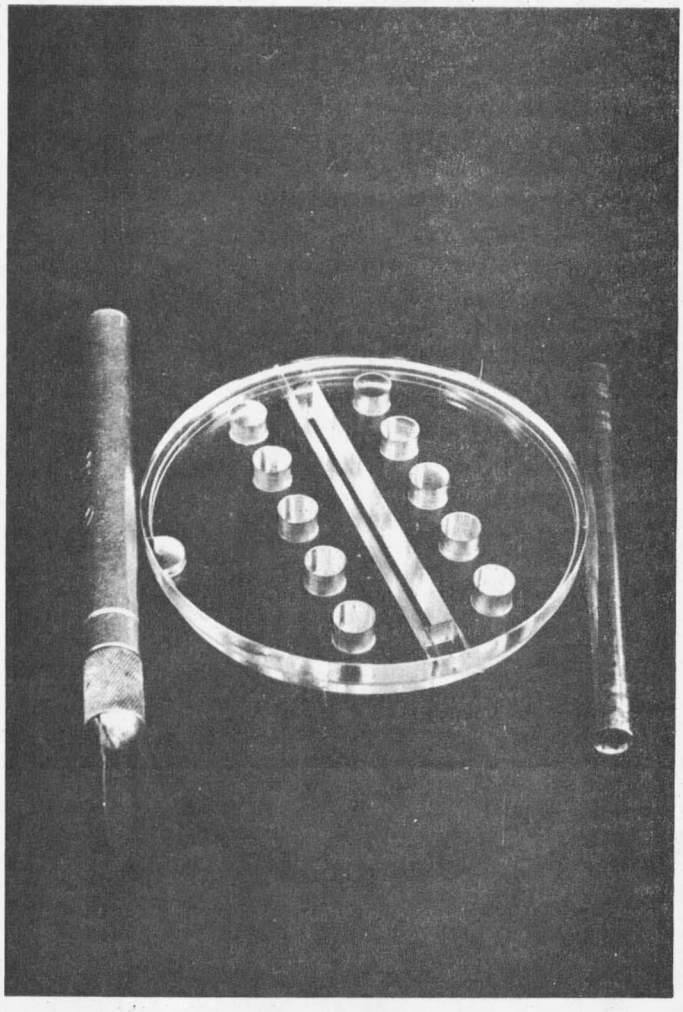


Fig. 3 Specially constructed template for use in spacing antigen wells and antiserum trench. The cork borer is used for cutting agar for antigen wells, and the X-acto knife for cutting the antiserum trench.

On the record card the individual plant number from which the antigens were expressed was entered inside the circle representing the well.

Following inoculation of the wells and trench with the appropriate antigens and antiserum, the plates were incubated at 21° C. for 48 hours, or at 21° C. for 24 hours plus an additional 48 hours at 4° C. During this incubation period the antigens and antiserum diffused through the gel and formed precipitate lines where specific antigen and antibody met in appropriate concentration. The plates were read at the end of the incubation period or stored at 4° C. for a few hours until they could be interpreted.

The gel-diffusion plates were read by allowing fluorescent light to pass through the agar at an oblique angle, thus making the precipitate lines clearly visible.

Precipitate lines that joined from one area of the plate to its adjacent area were interpreted as being formed by identical reactants; that is, the antigen which reacted with the antibody to form the joined precipitate lines were identical. The formation of a spur rather than a smooth curve where the lines joined was interpreted to mean that non-identical but similar antigens were reacting with the same antibody. Lines which did not join, even though they were close enough to do so, were considered to be different, and hence

different reacting antigens. The total number of lines, mode of joining of adjacent lines, and the spacing of lines were all given consideration.

Theoretically no antigen can react to produce more precipitate lines than that of the species used to stimulate antibody formation. It may produce as many, but probably less. Each species checked was plated next to the specific antigen to determine how many antigens they had in common. The greater the number of antigens in common the closer the relationship was considered to be.

One method of determining the serological relationship of different species, termed here the Four-way test, consisted of allowing each species to react with each of 4 different antisera--those produced in response to injected A. cristatiforme (#10-3), A. triticeum (#125-S), A. dasystachyum (#147-S, and A. panormitanum (#50a-7). The number of precipitate lines formed for each was recorded. In every case the readings were confirmed by Mr. John Ericson, a graduate student at Montana State College, who is also working on the serological relationships in Agropyron.

To illustrate the operation of the Four-way test, an example follows. When A. cristatiforme was allowed to react with its antiserum, 17 precipitate lines were produced. When A. triticeum reacted with its antiserum, 12 lines were produced. A. dasystachyum produced 9 lines when allowed to react with its antiserum. The reaction of A. panormitanum with its antiserum produced 12 lines. This is interpreted to mean that no species could produce any more than a total

of 50 lines when allowed to react with all four antisera. The number of lines produced with each reaction was listed separately as 14-7-8-6 respectively (arranged in this order in Table III). This was done for each species and all were compared for differences. For example, if a species produced lines such as 9-8-9-8, it would differ from the above reading by a total of 9 lines. This difference in lines was divided by 50 to put it on a basis of 1, and this is referred to as a difference index (0.18 for this example). If species differed by an index of less than 0.12, they were considered closely related. Table III (in results) was constructed so that each species could be checked for its minimum relationship to others used in this study. A difference index of 0, therefore, meant the plants being compared produced the same number of precipitate lines when reacting with the four antisera mentioned above. Failure to recognize the presence of lines would result in an error in the difference index. Species which had low index numbers when compared to one another were given additional tests.

The crested wheatgrasses were treated in considerably more detail than the other species because they are a taxonomically difficult group. Seven morphological forms of 6 species were considered serologically, morphologically, and cytologically. The serology consisted of absorbing antiserum produced in response to injected antigens from diploid A. cristatiforme (#10-3) with antigens from each of the other species. Antigen absorption consisted of adding antigens to antiserum until no further precipitation occurred. The mixture was mildly shaken

at frequent intervals for about 2 hours at room temperature. The absorbed antiserum was centrifuged at 3000 rpm. for one hour before use to remove precipitate. To facilitate interpretation of data, a series of plates was made differing only in the material used to absorb the antiserum.

A precipitate line was expected to form for each antigen which each species had in common with A. cristatiforme, but which the species used for absorption did not have. The test was considered invalid if any precipitate lines formed between the well containing the antigen used to absorb and the antiserum trench.

Scaled drawings of a glume, lemma, palea, and anther of each species of crested wheatgrass were made. The drawings were made from spikelets and florets in which pollen had formed but anthesis had not yet occurred. The drawings are not intended to indicate an average plant, but only a selected plant of each species. In all cases the lemma, palea, and anther came from the lowermost floret of the spikelet from which the glume was taken. The spikes were taken from plants growing in the field nursery in the summer of 1963. The parts were measured while observing them through a binocular dissecting microscope. The spikelets were softened with water for ease of handling during measuring and drawing.

Since some investigators have speculated that A. sibiricum is of hybrid origin, a series of modified gel-diffusion plates were designed to

test this hypothesis. The plates contained only 3 antigen wells on both sides of the antiserum trench. For this test, antiserum was produced to antigens of A. sibiricum (#68-5). The antigens of the species of supposedly hybrid origin, A. sibiricum, and the two supposed parent species, A. repens and A. cristatiforme, were plated in such a way that the antigens from A. sibiricum were located between the other two. The total number of precipitate lines formed for each species was counted and their relations, as indicated by their joining or crossing, recorded. The supposed hybrid should have all antigens found in both of the supposed parents. Unique reactions, that is--the formation of precipitate lines for A. sibiricum only, would indicate the presence of a hybrid antigen or that A. sibiricum was not a product of the supposed cross.

Tests were run in duplicate, in most cases in quadruplicate.

EXPERIMENTAL RESULTS

I. SEROLOGY

(a) Four-way Test

In this work the testing of antigens of a species with four different antisera is termed a Four-way test. Each species was compared with all other species investigated and the results are given as difference indexes in Table III. Methods for determining the difference index for a species have already been described. For every comparison of two plants a difference index is interpreted as indicative of their serological relationship--the lower the difference index, the closer the serological relationship. For purposes of interpretation a difference index of 0.12 was selected as a standard of reference. The crested wheatgrass complex, a homogeneous group, is clearly morphologically distinct from the other Agropyron species. The so-called species of this group are considered to be closely related because those of the same ploidy level freely hybridize and produce fertile offspring (Knowles, 1955). The greatest difference index of any two crested wheatgrasses tested was 0.12; this was the basis for selection of the standard of reference. Any difference index of 0.12 or less was interpreted as expressing close serological similarity.

Results of the Four-way test indicated that in some cases species may show a close, or even identical, serological relationship when by other criteria they are different. For example, when A. brachyphyllum (#3-2) was compared with A. donianum (#32-1), they both produced the same number of precipitate lines in reaction to each of the four antisera (12-8-8-8),

Table III(Part 1). Data of the "four-way" tests, difference indexes and summation indexes.

Precipitate lines formed	Species No.	0.5-2	1-5	2-8	3-2	4a-1	9-9	10-3	28-9	30-9	32-1	33-3	35a-2	36-5	37-5	44-6	46a-2	47-3	48b-1	50a-7	57-5	62a-2
14-7-9-11	0.5-2	8.02	.20	.22	.14	.28	.14	.12	.14	.08	.14	.14	.20	.14	.12	.18	.18	.14	.14	.10	.18	.16
11-6-8-6	1-5	0	6.94	.18	.10	.16	.14	.28	.18	.24	.10	.18	.16	.10	.12	.02	.18	.06	.22	.26	.06	.04
7-7-7-9	2-8		0	8.38	.16	.14	.24	.30	.28	.26	.16	.20	.10	.12	.18	.16	.24	.20	.28	.24	.12	.14
12-8-8-8	3-2			0	5.14	.14	.08	.18	.12	.14	0	.08	.10	.04	.02	.08	.12	.08	.12	.16	.08	.06
9-9-6-7	4a-1				0	7.22	.14	.28	.22	.28	.14	.18	.08	.14	.16	.14	.10	.18	.22	.22	.14	.12
13-9-9-7	9-9						0	.14	.08	.18	.08	.08	.14	.12	.06	.12	.08	.08	.08	.16	.16	.10
17-9-9-10	10-3						6.34	0	.10	.08	.18	.14	.20	.22	.16	.26	.18	.22	.06	.14	.26	.24
16-8-9-7	28-9							9.54	0	.10	.12	.16	.22	.16	.10	.16	.12	.12	.04	.20	.20	.14
16-8-8-11	30-9								7.58	0	.14	.14	.20	.18	.12	.22	.18	.18	.10	.10	.22	.20
12-8-8-8	32-1									8.50	0	.14	.20	.18	.12	.22	.18	.18	.10	.10	.22	.20
13-10-8-9	33-3										5.14	.08	.10	.04	.02	.08	.12	.08	.12	.16	.08	.06
10-9-7-9	35a-2											0	.10	.12	.06	.16	.12	.12	.12	.12	.18	.14
12-7-7-8	36-5												0	.10	.12	.14	.14	.18	.18	.14	.10	.12
13-8-8-8	37-5													0	.06	.08	.12	.08	.16	.16	.04	.06
11-7-8-6	44-6													5.38	0	.10	.10	.06	.10	.14	.10	.08
14-9-6-7	46a-2														5.22	0	.16	.04	.20	.24	.08	.02
13-7-8-6	47-3															6.34	0	.12	.12	.12	.16	.14
16-9-9-8	48b-1																6.58	0	.16	.20	.12	.06
14-9-7-12	50a-7																	6.34	0	.16	.20	.18
11-6-7-8	57-5																		7.74	0	.20	.22
11-7-8-7	62a-2																			8.18	0	.06
10-7-7-8	64-8																				6.08	0
17-8-9-7	68-5																					5.62

64-8	68-5	75-3	80a-2	93-4	104-3	111-12	112-5	115-16	121-4	122-2	125-S	128-S	137-5	138-3	140-3	141-S	145-S	25-9	A1-S	146-S	147-S	148-S	149-S
.18	.16	.12	.08	.24	.12	.18	.18	.26	.22	.08	.20	.40	.18	.32	.28	.22	.20	.08	.14	.16	.04	.30	.34
.10	.20	.20	.24	.12	.12	.14	.10	.18	.14	.16	.24	.20	.14	.16	.16	.06	.12	.24	.18	.12	.20	.14	.14
.08	.30	.22	.26	.18	.18	.16	.16	.08	.08	.18	.34	.18	.08	.14	.18	.16	.14	.26	.24	.06	.22	.16	.20
.08	.14	.10	.14	.10	.02	.12	.04	.16	.12	.06	.18	.30	.08	.22	.14	.12	.06	.14	.08	.10	.10	.20	.24
.10	.24	.20	.28	.08	.16	.18	.10	.10	.06	.20	.24	.20	.10	.12	.08	.10	.08	.28	.18	.12	.24	.10	.14
.16	.10	.10	.18	.10	.06	.20	.08	.24	.20	.06	.10	.34	.16	.26	.18	.16	.14	.18	.08	.18	.10	.24	.28
.26	.08	.12	.08	.24	.16	.26	.18	.34	.30	.12	.16	.48	.22	.40	.32	.30	.24	.08	.14	.24	.08	.38	.42
.20	.02	.14	.10	.18	.10	.24	.16	.28	.24	.10	.10	.38	.20	.30	.22	.20	.18	.10	.12	.22	.10	.28	.32
.22	.12	.12	0	.24	.12	.22	.18	.30	.26	.12	.20	.44	.18	.36	.28	.26	.20	0	.14	.20	.08	.34	.38
.08	.14	.10	.14	.10	.02	.12	.04	.16	.12	.06	.18	.30	.08	.22	.14	.12	.06	.14	.08	.10	.10	.20	.24
.16	.18	.02	.14	.10	.06	.16	.08	.24	.20	.06	.14	.38	.12	.30	.22	.20	.14	.14	.04	.14	.10	.28	.32
.06	.24	.12	.20	.08	.12	.14	.06	.14	.10	.12	.24	.28	.02	.20	.12	.14	.08	.20	.14	.04	.16	.18	.22
.04	.18	.14	.18	.10	.06	.08	.08	.12	.08	.10	.22	.26	.08	.18	.14	.08	.06	.18	.12	.06	.14	.16	.20
.10	.12	.08	.12	.12	0	.14	.06	.18	.14	.04	.16	.32	.10	.24	.16	.14	.08	.12	.06	.12	.08	.22	.26
.08	.18	.18	.22	.10	.10	.16	.08	.16	.12	.14	.22	.22	.12	.18	.14	.08	.10	.22	.16	.10	.18	.16	.16
.16	.14	.10	.18	.10	.10	.16	.12	.20	.16	.14	.14	.30	.16	.22	.14	.12	.10	.18	.08	.18	.14	.20	.24
.12	.14	.14	.18	.14	.06	.16	.12	.20	.16	.10	.18	.26	.16	.22	.18	.12	.14	.18	.12	.14	.14	.20	.20
.20	.06	.10	.10	.18	.10	.24	.12	.28	.24	.10	.10	.42	.20	.34	.26	.24	.18	.10	.08	.22	.10	.32	.36
.20	.22	.10	.10	.18	.14	.20	.16	.28	.24	.14	.22	.42	.16	.34	.26	.24	.18	.10	.12	.18	.10	.32	.36
.04	.22	.18	.22	.10	.10	.08	.08	.12	.08	.14	.26	.22	.08	.14	.14	.04	.06	.22	.16	.06	.18	.12	.16
.06	.16	.16	.20	.08	.08	.14	.06	.14	.10	.12	.20	.24	.10	.16	.12	.06	.08	.20	.14	.08	.16	.14	.18
0	0																					
5.86	.22	.18	.22	.10	.10	.12	.08	.08	.04	.14	.26	.22	.04	.14	.10	.08	.06	.22	.16	.02	.18	.12	.16
	0																					
	8.42	.16	.12	.20	.12	.26	.18	.30	.26	.12	.12	.40	.22	.32	.24	.22	.20	.12	.14	.24	.12	.30	.34

Table III (Part 2). Data of the "four-way" tests, difference index and summation indexes.

Precipitate lines formed	Species No.	75-3	80a-2	93-4	104-3	111-12	112-5	115-16	121-4	122-2	125-S	128-S	137-5	138-3	140-3	141-S	145-S	25-9	A1-S	146-S	147-S	148-S	149-S
		0																					
14-10-8-9	75-3	6.98	.12	.12	.08	.18	.10	.26	.22	.08	.12	.40	.14	.32	.24	.22	.16	.12	.02	.16	.08	.30	.34
			0																				
16-8-8-11	80a-2		8.50	.24	.12	.22	.18	.30	.26	.12	.20	.44	.18	.36	.28	.26	.20	0	.14	.20	.08	.34	.38
				0																			
11-10-7-7	93-4			6.54	.12	.18	.06	.18	.14	.16	.16	.28	.10	.20	.12	.10	.08	.24	.10	.12	.20	.18	.22
					0																		
13-8-8-8	104-3				5.22	.14	.06	.18	.14	.04	.16	.32	.10	.24	.16	.14	.08	.12	.06	.12	.08	.22	.26
						0																	
12-6-5-9	111-12					7.34	.16	.12	.12	.14	.30	.22	.12	.14	.14	.08	.10	.22	.20	.10	.18	.12	.16
							0																
11-9-8-8	112-5						5.58	.16	.12	.10	.18	.30	.08	.22	.14	.12	.06	.18	.08	.10	.14	.20	.24
								0															
8-7-58	115-16							8.26	.04	.22	.34	.14	.12	.06	.10	.12	.10	.30	.24	.10	.26	.08	.12
									0														
9-7-6-8	121-4								6.86	.18	.30	.18	.08	.10	.10	.08	.06	.26	.20	.06	.22	.08	.12
										0													
13-8-9-9	122-2									6.10	.16	.36	.10	.28	.20	.18	.12	.12	.10	.12	.04	.26	.30
											0												
15-12-9-7	125-S										9.74	.44	.26	.36	.28	.26	.24	.20	.10	.28	.16	.34	.38
												0											
7-4-4-6	128-S											13.06	.26	.08	.16	.18	.24	.44	.38	.24	.40	.10	.06
													0										
10-8-7-9	137-5												5.98	.18	.10	.12	.06	.18	.16	.02	.14	.16	.20
														0									
8-5-5-7	138-3													9.82	.12	.10	.16	.36	.30	.16	.32	.02	.06
															0								
10-8-4-7	140-3														7.74	.10	.08	.28	.22	.12	.24	.10	.14
																0							
11-6-6-7	141-S															6.66	.06	.26	.20	.10	.22	.08	.12
																	0						
11-8-6-8	145-S																5.66	.20	.14	.08	.16	.14	.18
																		0					
16-8-8-11	25-9																	8.50	.14	.20	.08	.34	.38
																			0				
14-10-88	A1-S																		6.66	.18	.10	.28	.32
																				0			
10-7-7-9	146-S																			6.18	.16	.14	.18
																					0		
14-8-9-10	147-S																				7.10	.30	.34
																						0	
9-5-5-7	148-S																					9.34	.04
																							0
9-4-5-6	149-S																						10.54

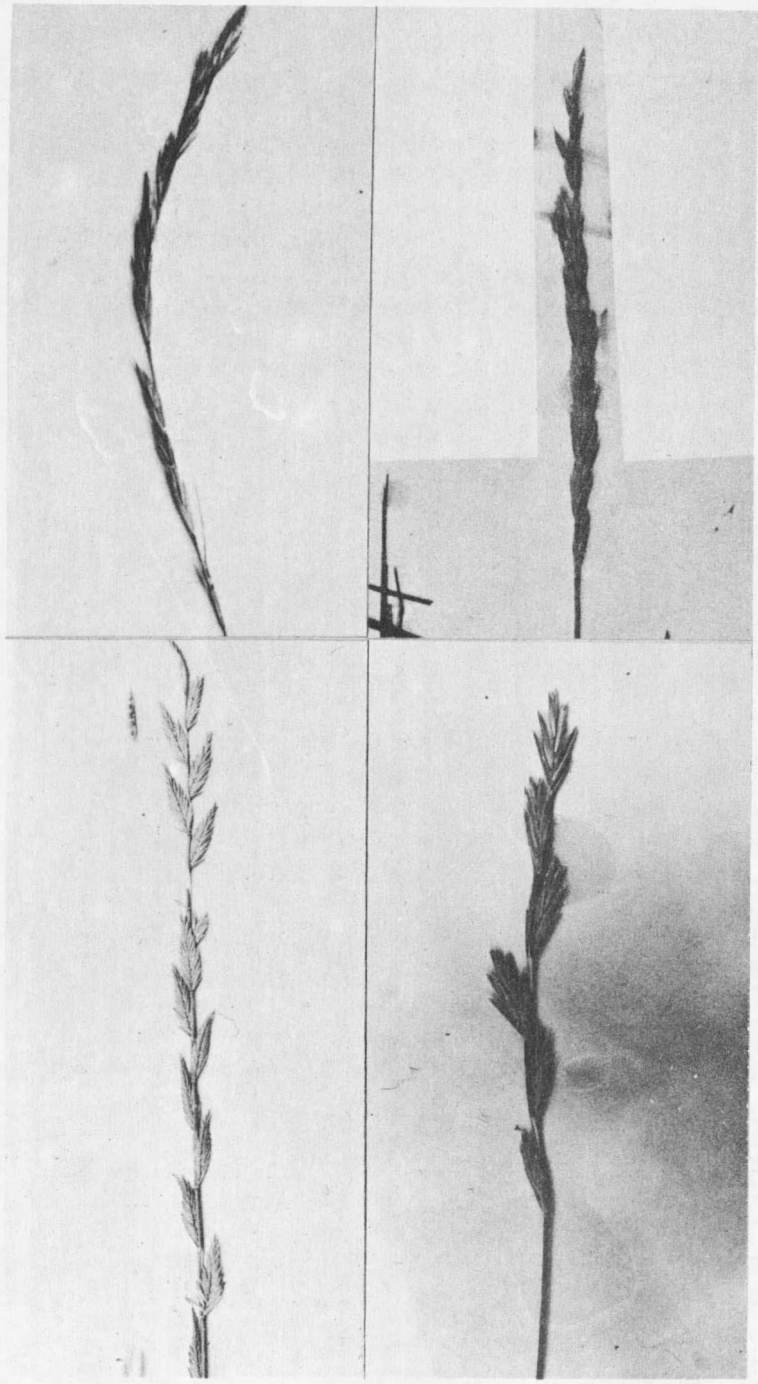
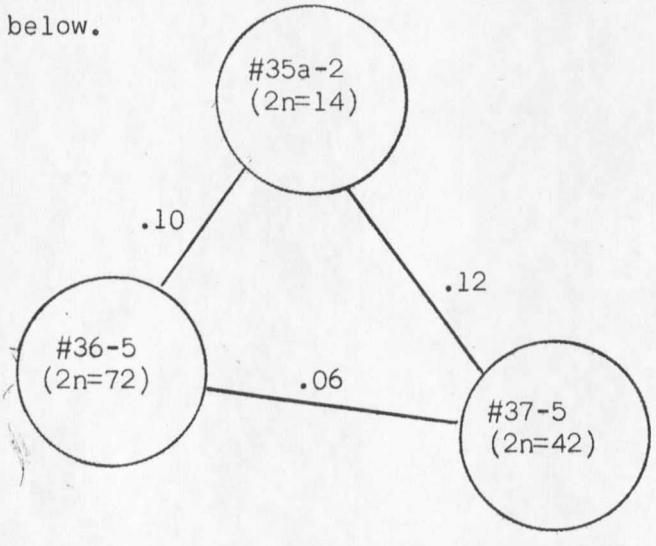


Fig. 4 Morphology of spikes--a) A. brachyphyllum (0.5X), b) A. donianum (0.5 X), c) A. elongatum, hexaploid (0.3X), d) A. trichophorum (1 X).

even though there were some noticeable morphological differences (Figure 4a,b).

As an additional example, the hexaploids, A. elongatum (#37-5) and A. trichophorum (#104-3) (Figure 4c,d), both considered valid species, had identical reactions with all four antisera. Each produced 13 lines when allowed to react with A. cristatiforme and 8 with A. triticeum, A. dasystachyum, and A. panormitanum antisera. Their difference index was zero. Hexaploid A. elongatum (#37-5) and A. trichophorum (#104-3) of the section Holopyron were found to be very closely related to A. brachyphyllum (3-2) and A. donianum (#32-1) of the section Goulardia, as indicated by Their difference index of 0.02. They differed only in their reaction to A. cristatiforme antiserum, and the difference was a single precipitate line. Other species relationships also showed a difference index of zero and will be presented later in the results of the serology of the crested wheatgrass group.

A diploid (#35a-2), a hexaploid (#37-5), and a decaploid (#36-5) strain of A. elongatum were compared in this study. Their difference indexes are shown below.



When these strains were compared with other species, some unexpected relationships were noted. For example, when strain #35a-2 was compared with A. littorale (#137-5), the difference index was 0.02. Each produced the same number of precipitrate lines in reaction with 3 of the 4 antisera, differing only in their reaction with antiserum of A. triticeum. Strain #35a-2 formed 9 precipitate lines while A. littorale formed only 8. Their difference index was 0.02, a closer serological relationship than it was observed for any two of the strains of A. elongatum (Figure 5).

Strain #35a-2 was found to be more closely related to other species than to other strains of A. elongatum. Strain #35a-2 (diploid A. elongatum) and A. repens (#146-S) had a low difference index of 0.04 and they differed only in their reaction to diploid A. triticeum antiserum. A. repens produced only 7 lines while strain #35a-2 produced 9. Comparison of A. repens and A. littorale (#137-5) showed that they differed only in their reaction to A. triticeum antiserum. A. repens produced 7 precipitate lines while A. littorale produced 8.

A. repens (#146-S) and the decaploid strain of A. elongatum (#36-5) had a similar relationship. They produced the same number of precipitate lines when allowed to react with antisera of A. triticeum and A. dasystachyum, but they differed in reaction to the two other antisera. A. elongatum (#36-5) formed 12 precipitate lines with A. cristatiforme antiserum, and 8 with A. panormitanum. A. repens formed 10 lines in reaction with A. cristatiforme antiserum and 9 with that of A. panormitanum. Their difference index was 0.06, a relationship as close

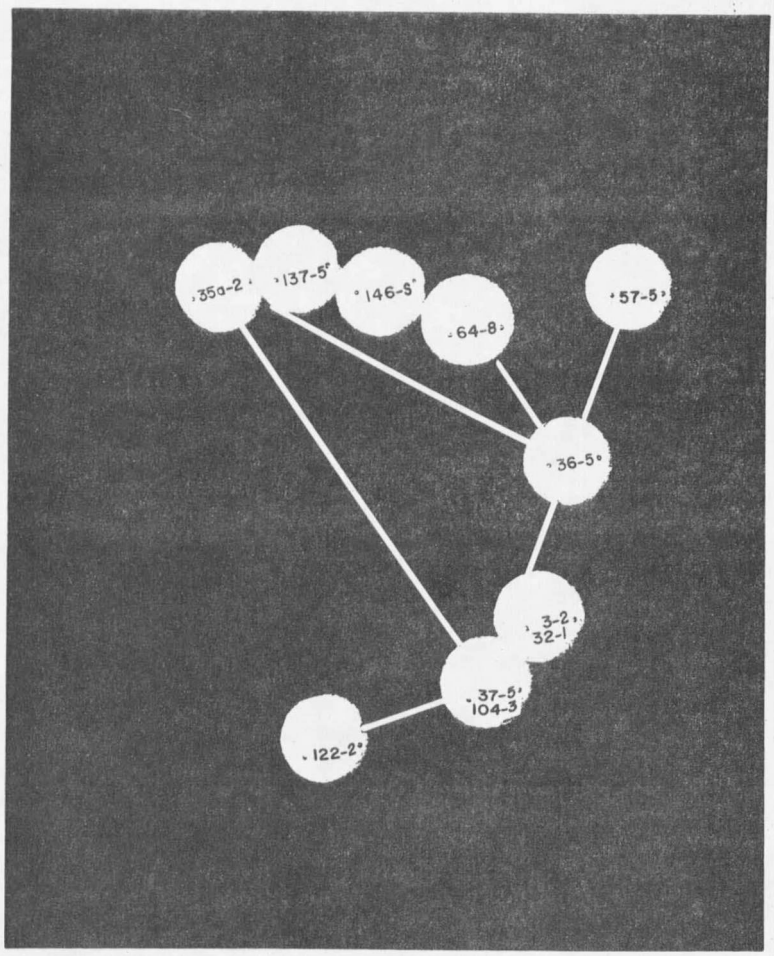


Fig. 5 Relative serological relationships of three strains of A. elongatum, #35a-2 (diploid), #37-5 (hexaploid), #36-5 (decaploid), and other closely related species.

as that between two of the strains of A. elongatum (#36-5 and #37-5).

Since some serological differences were found in the different ploidy levels of A. elongatum and since Dewey (1963c) has suggested that the crested wheatgrass complex may actually represent a polyploid series of the same species rather than different species, the relationships between the crested wheatgrasses were tested. They had difference indexes ranging from zero to 0.12 when compared to each other (Table IV). The two strains of A. desertorum (#s 25-9 and 30-9), when compared with each other and with A. cristatum (#80a-2) by the Four-way test, had difference indexes of zero indicating that each formed the same number of precipitate lines as the others in reaction with the 4 different antisera. Each formed 16 precipitate lines in reaction with A. cristatiforme antiserum, 8 with A. triticeum antiserum, 11 with A. panormitanum antiserum, and 8 with A. dasystachyum antiserum. Data from Table IV (taken from Table III) indicate that A. sibiricum (#68-5) differed from A. desertorum (both strains, #25-9 and #30-9) and A. cristatum (#80a-2) by a difference index of 0.12. All other comparisons of crested wheatgrasses had difference indexes of less than 0.12. The crested wheatgrasses formed a serologically and morphologically distinct group.

It was not less interesting to find other species forming clusters or subgroups, which suggests recent speciation or evolution from a common ancestor. One of these subgroups made obvious by the Four-way test had A. intermedium (#44-6) as the central species. It had a difference index of 0.02 in comparison with both A. scabriglume (#1-5) and

Table IV. Difference indexes in comparison of the crested wheatgrasses (section Agropyron).

Species	MSC Field No.	10-3	80a-2	25-9	30-9	28-9	48b-1	68-5
A. cristatiforme	10-3	0	0.08	0.08	0.08	0.10	0.06	0.08
A. cristatum	80a-2		0	0	0	0.10	0.10	0.12
A. desertorum	25-9			0	0	0.10	0.10	0.12
A. desertorum	30-9				0	0.10	0.10	0.12
A. imbricatum	28-9					0	0.04	0.02
A. mongolicum	48b-1						0	0.06
A. sibiricum	68-5							0

A. riparium (#62a-2), and 0.04 when compared with A. latiglume (#47-3). A. scabriglume and A. riparium had a difference index of 0.04 when compared with each other, and both were serologically related to A. latiglume by a difference index of 0.06. The relative serological relationships of the species of this group are represented by the model in Figure 6.

Another cluster, A. tsukushiense (#148-S), A. mayebaranum (#149-S), A. scabrifolium (#138-3), and A. pycnanthum (#128-S), formed a rather closely knit group which was serologically very different from the majority of the species studied (Figures 7 and 20). A. mayebaranum had a difference index of 0.06 with A. pycnanthum, 0.04 with A. tsukushiense, and 0.06 with A. scabrifolium. A. scabrifolium was related to A. pycnanthum and A. tsukushiense by difference indexes of 0.08 and 0.02, respectively. A. tsukushiense and A. pycnanthum had a difference index of 0.10, the highest for this cluster.

Even though A. pycnanthum (#128-S) had a close relationship to the other 3 species in its cluster, it differed from all others tested by a difference index of 0.14 or more. Eleven of the 45 strains of 41 species investigated differed from A. pycnanthum by a difference index of 0.40 or more, 22 differed by 0.30 or more, and 37 differed by 0.20 or more. A. pycnanthum differed from A. cristatiforme (#10-3) by a difference index of 0.48---the largest difference index observed in this investigation.

One of the most surprising serological relationships revealed by this Four-way test was that between the old world annual A. hirsutum

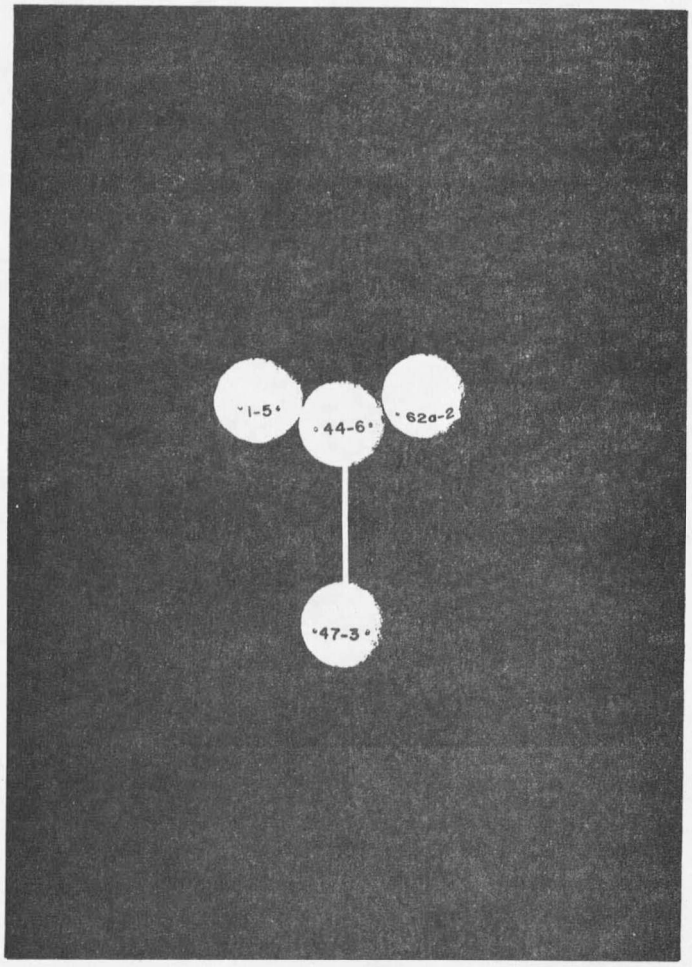


Fig. 6 A species cluster with A. intermedium (#44-6) as the central species; others are #1-5, A. scabriglume; #62a-2, A. riparium; and #47-3, A. latiglume. The relative distances are indicative of serological affinity.

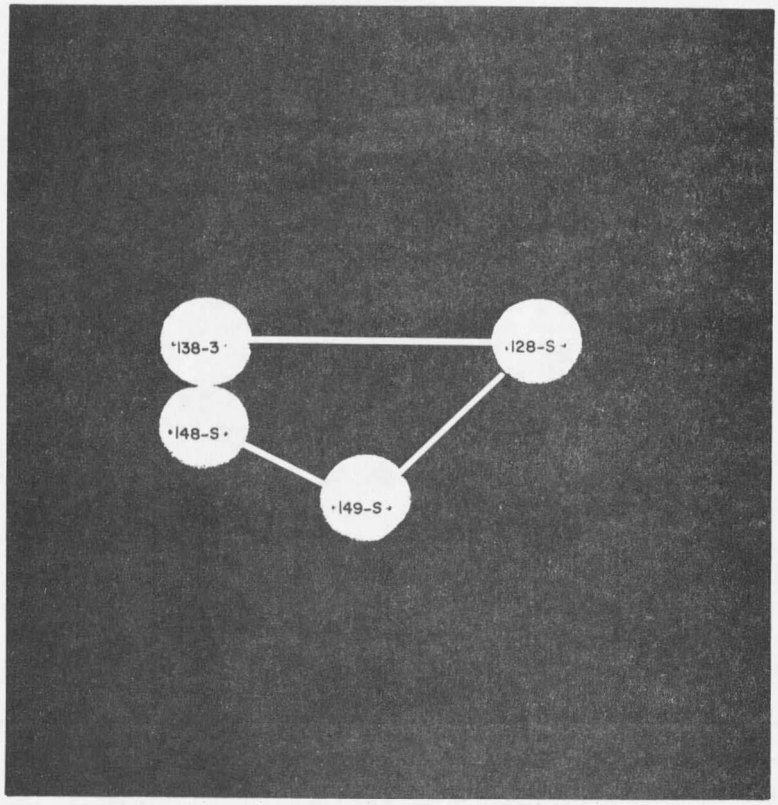


Fig. 7 A cluster of serologically aberrant species; #128-S, A. pycnanthum; #138-3, A. scabrifolium; #148-S, A. tsukushiense; and #149-S, A. mayebaranum.

(#A1-S), section Eremopyrum, and the new world perennial A. spicatum (#75-3) which is in the section Holopyron. These species are very different in morphology (Figure 8). They had a difference index of 0.02, differing only in their reaction to antiserum of A. panormitanum; A. spicatum formed 9 lines while A. hirsutum formed only 8. Their reactions to the other 3 antisera were identical (14-10-8). A. spicatum also had a difference index of 0.02 when compared to A. elongatiforme (#33-3), but this was not unexpected since they are in the same section. Comparison of A. hirsutum and A. elongatiforme resulted in a difference index of 0.04, establishing that A. spicatum was serologically intermediate between A. hirsutum and A. elongatiforme (Figure 9).

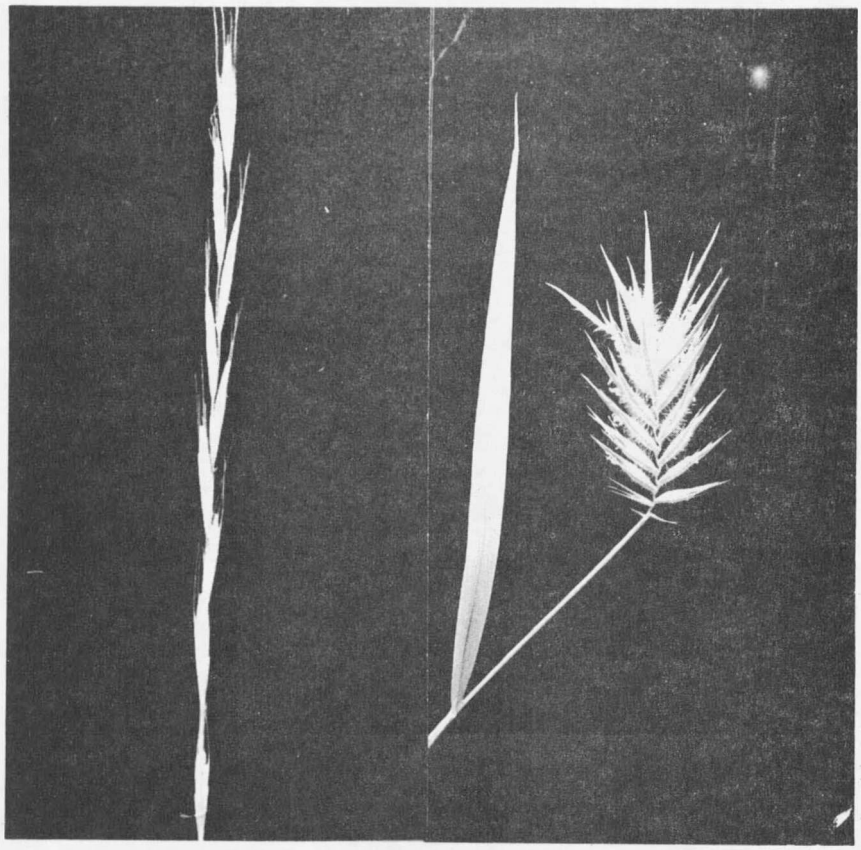


Fig. 8 A new world perennial, a) A. spicatum, section Holopyron, and an old world annual, b) A. hirsutum, section Eremopyrum, which are very closely related serologically.

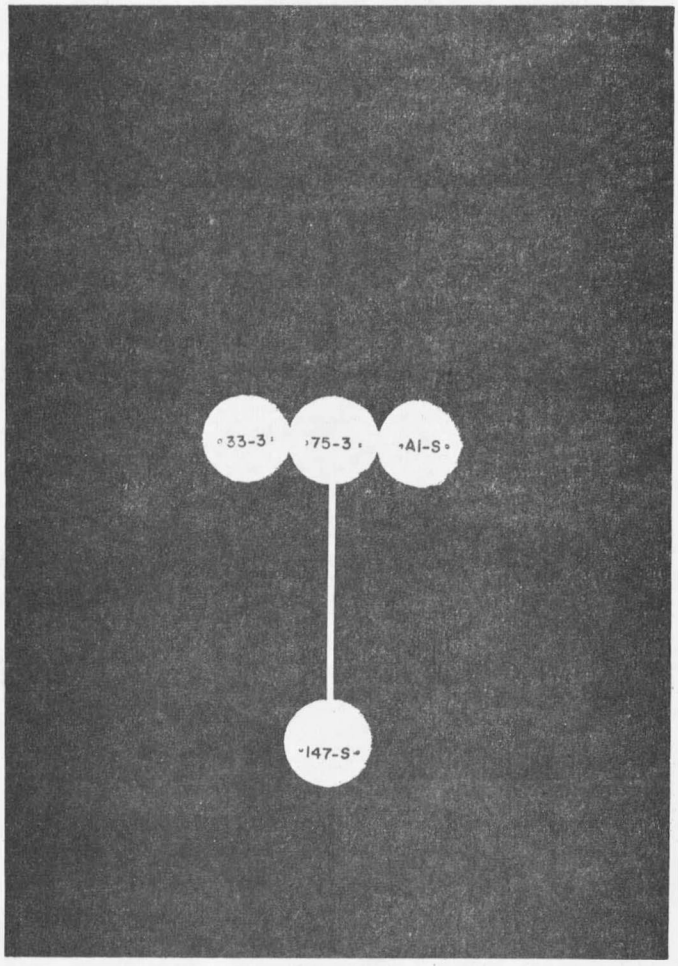


Fig. 9 A cluster of species showing the relative serological affinities of #33-3, A. elongatiforme (section Holopyron); #75-3, A. spicatum (section Holopyron); #A1-S, A. hirsutum (section Eremopyrum); and #147-S, A. dasystachyum (section Holopyron).

(b) Antigen Absorption and Adjacent Plating

In view of the fact that the Four-way test gave a minimum difference (when, in fact, the actual difference may be somewhat greater than indicated by the difference index), an additional test was carried out to find actual differences. In some instances species had a difference index of zero when compared in the Four-way test. For example, they had the same number of lines formed with the different antisera, such as 13-8-8-8 for species A, and 13-8-8-8 for species B. Some of these lines may not have been identical in nature. To detect the true relationships, the absorption technique was used to remove those antibodies that react with the antigens of a certain species. In all cases in which the difference index was zero the absorption test was used. Some comparisons resulting in a difference index of 0.02 were also given the absorption test, especially if the two species were in different sections.

The difference index of A. donianum (#32-1) compared with A. brachyphyllum (#3-2) was zero in spite of the fact that some morphological differences exist and that they are considered valid species. The absorption method was employed and the results were consistent with the findings in the Four-way test. Antiserum of A. sibiricum was absorbed with antigens of A. brachyphyllum and both A. brachyphyllum and A. donianum antigens were plated against it for any possible antigen-antibody reactions. The plates showed no visible precipitate lines. In a further check the antiserum was absorbed with antigens of A. donianum and the two species were plated. Again no precipitate

lines were observed (Figure 10). In the Four-way test the extracts of these two species had identical reactions with all four antisera (12-8-8-8); as a result each line formed for one species smoothly joined one of the other. When checked with 5 different sources of antisera the serological reactions were the same.

Another comparison of two species which had a difference index of zero was that of A. elongatum (#37-5, hexaploid), and A. trichophorum (#104-3, hexaploid). Antiserum produced in response to antigens of A. sibiricum (#68-5) was absorbed with antigens of A. elongatum. The antigens of the two species were then plated in adjacent wells and allowed to react with diffusing antibodies from the central trench. After 48 hours of incubation at 21° C. no precipitate lines were observed (Figure 10, see legend). The same two species were again plated as above and allowed to react with antiserum which had been absorbed with antigens of A. trichophorum (#104-3). Careful study of the plates after an incubation period of slightly more than 48 hours revealed that no precipitate lines had formed.

Data from Table III show that A. brachyphyllum (#3-2) and A. donianum (32-1) differed from A. elongatum (#37-5) and A. trichophorum (#104-3) by a difference index of 0.02 which shows a close relationship. Plates were designed in which antigens of these four species and those of A. sibiricum were plated and allowed to react with A. sibiricum antiserum absorbed with extracts from A. elongatum (#37-5). After the proper incubation time for the plates no lines were visible

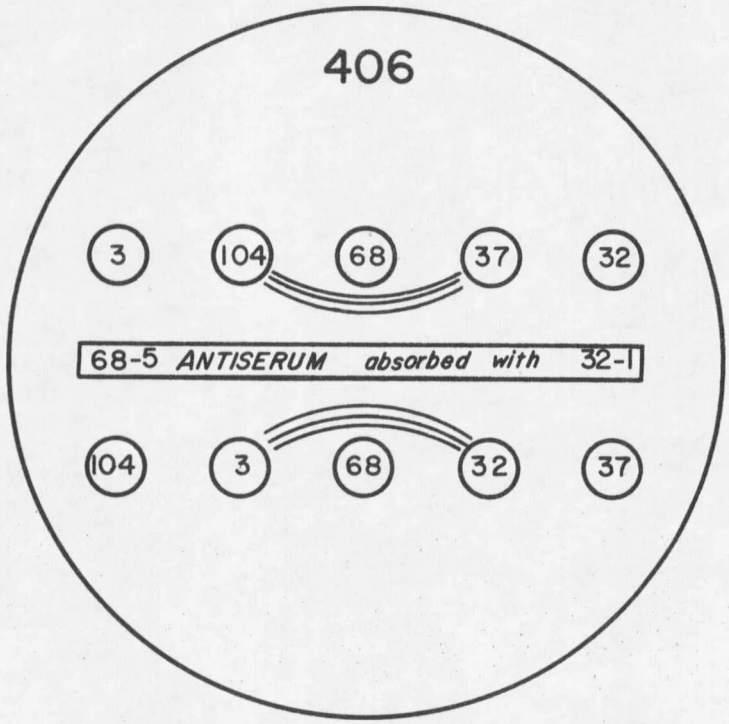


Fig. 10 A gel-diffusion plate in which A. sibiricum (#68-5) antiserum was absorbed with antigens of A. donianum (#32-1). When, on other plates, the antiserum was absorbed with A. brachyphyllum (#3-2), A. elongatum (#37-5), or A. trichophorum (#104-3), the results were exactly as shown here, that is, each completely removed all antibodies with which the others could react.

except for the specific, A. sibiricum, where a number of lines developed.

Additional plates, of the same design and antigen placement, were made and the antiserum was absorbed with A. brachyphyllum (#3-2), A. donianum (#32-1), and A. trichophorum (#104-3) extracts. In all cases the results were identical to those obtained when the antiserum was absorbed with the antigens of A. elongatum (#37-5)--that is, no precipitate lines appeared except by the well containing antigens from A. sibiricum.

The antigen absorption concerned with the crested wheatgrasses, some of which had difference indexes of zero, was done using antiserum produced in response to antigens of A. cristatiforme (#10-3). This antiserum lot was not the same as that used in the Four-way test. It was produced by the same rabbit, but the antiserum used in the Four-way test was taken following additional injections.

When the antiserum was absorbed with antigens from A. mongolicum (#48b-1), a single precipitate line appeared by the antigen wells of A. imbricatum (#28-9) and A. cristatiforme (#10-3), indicating that these two species had one antigen which A. mongolicum did not have. No precipitate lines appeared by the antigen wells of A. desertorum (#25-9 and #30-9), A. cristatum (#80a-2), and A. sibiricum (#68-5). As expected, no lines appeared by the antigen well of A. mongolicum, indicating complete absorption (Figure 11b).

When the A. cristatiforme antiserum was absorbed with antigens from A. imbricatum (#28-9), a single precipitate line appeared by the antigen wells of A. cristatiforme (#10-3) and A. mongolicum

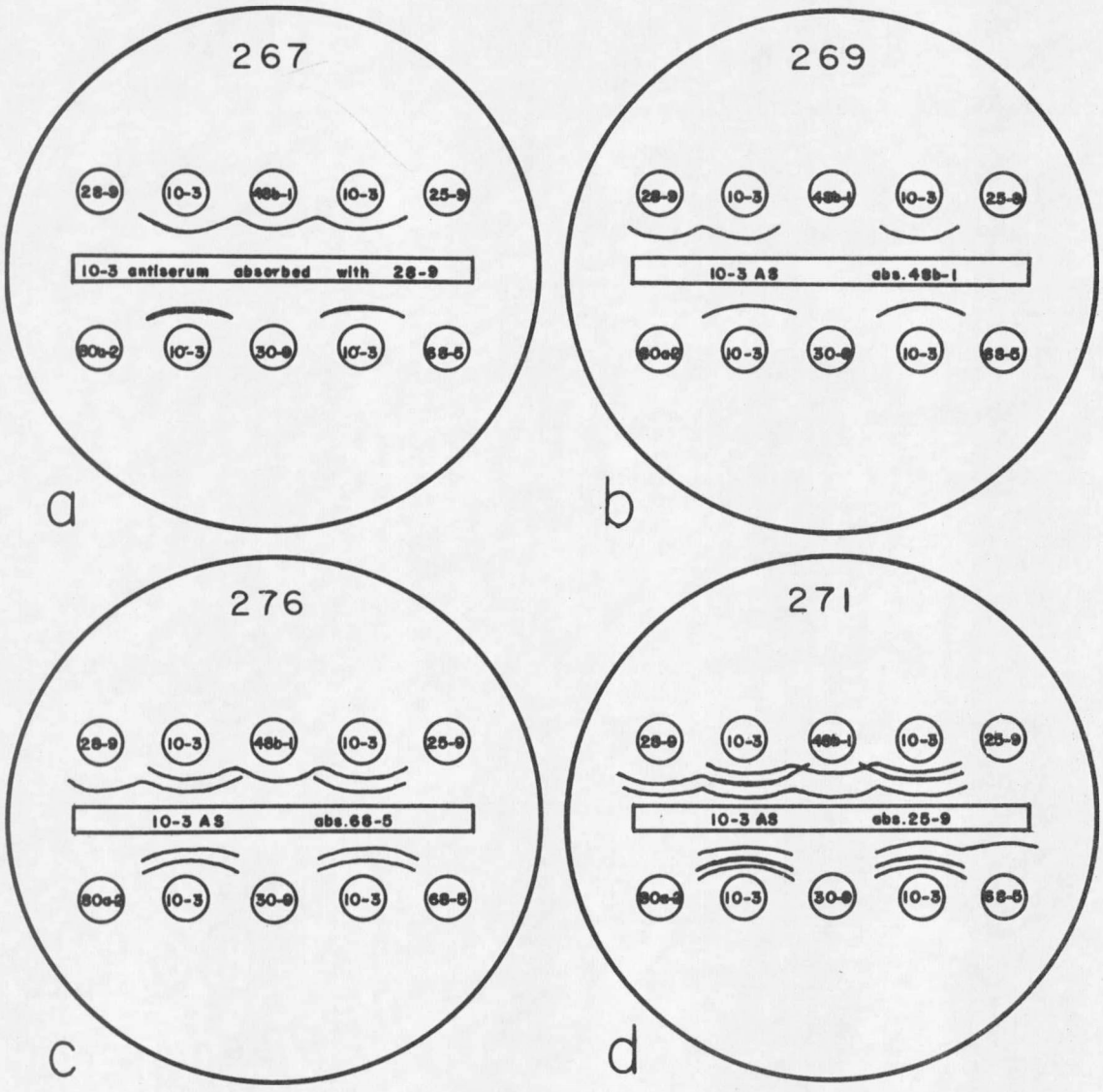


Fig. 11 Absorption plates of the crested wheatgrasses. The antiserum was absorbed as indicated in the drawings. When the antiserum was absorbed with antigens of A. desertorum (#30-9) and A. cristatum (#80a-2) the results were exactly as shown in d above.

(#48b-1). This shows that these two species contain an antigen which is not present in A. imbricatum. No lines were formed by wells containing antigens of the two strains of A. disertorum (#80a 2), and A. sibiricum (#68-5), indicating that these species had at least as many antigens in common with A. cristatiforme as A. imbricatum did. No lines appeared near the well of A. imbricatum, indicating complete absorption (Figure 11a).

Absorption of the antiserum (#10-3) by antigens of A. sibiricum (#68-5) left two lines by the antigen well of A. cristatiforme (#10-3). One of the lines was in common with a line formed by the A. imbricatum well and the other in common with a precipitate line left by the A. mongolicum well (Figure 11c). No other lines appeared on this plate. Absorption was complete.

When the A. cristatiforme antiserum was absorbed with antigens from A. desertorum (#25-9), three lines were visible by the A. cristatiforme well, two by A. imbricatum, two by A. mongolicum, and one by A. sibiricum. The line by the A. sibiricum well was the same as one of those by the well of A. imbricatum and A. mongolicum. The second lines formed by A. imbricatum and A. mongolicum were not in common with each other but were in common with one of those of A. cristatiforme (Figure 11d). No other lines appeared on the plate.

When the antiserum was absorbed with antigens of A. desertorum (#30-9) and A. cristatum (#80a-2), the absorption pattern was identical with that of A. desertorum (#25-9) mentioned in the paragraph above and shown in Figure 11d.

The close serological relationship of the Eurasian annual, A. hirsutum (#A1-S), section Eremopyrum, and the new world perennial A. spicatum (#75-3), section Holopyron, was given an additional test. The supply of crude extract from A. hirsutum was very small due to the scarcity of plants available and their small size. For this reason adjacent plating was used rather than absorption. Results of plating the antigens of the two species in adjacent wells and allowing them to react with antiserum produced in response to A. sibiricum antiserum showed that all precipitate lines were in common except for a single one formed by A. spicatum extract. The precipitate lines joined by a smooth curve indicating identical reactions. The two species were also plated in like manner and allowed to react with A. panormitanum antiserum. The results were consistent with the reaction with A. sibiricum antiserum; that is, A. spicatum had one antigen in common with A. sibiricum and A. panormitanum which the annual A. hirsutum did not have (Figure 12). The results also agreed with the reactions of the Four-way test.

This method of adjacent plating was also used to determine whether A. sibiricum was of hybrid origin resulting from a natural cross of A. repens and A. cristatiforme. The three species were plated, with A. sibiricum between A. repens and A. cristatiforme, and allowed to react with antiserum produced in response to A. sibiricum. All lines formed for A. repens were in common with those of A. sibiricum, and all those of A. cristatiforme were also in common with the specific. Of considerable interest was the observation that A. sibiricum had an antigen-antibody

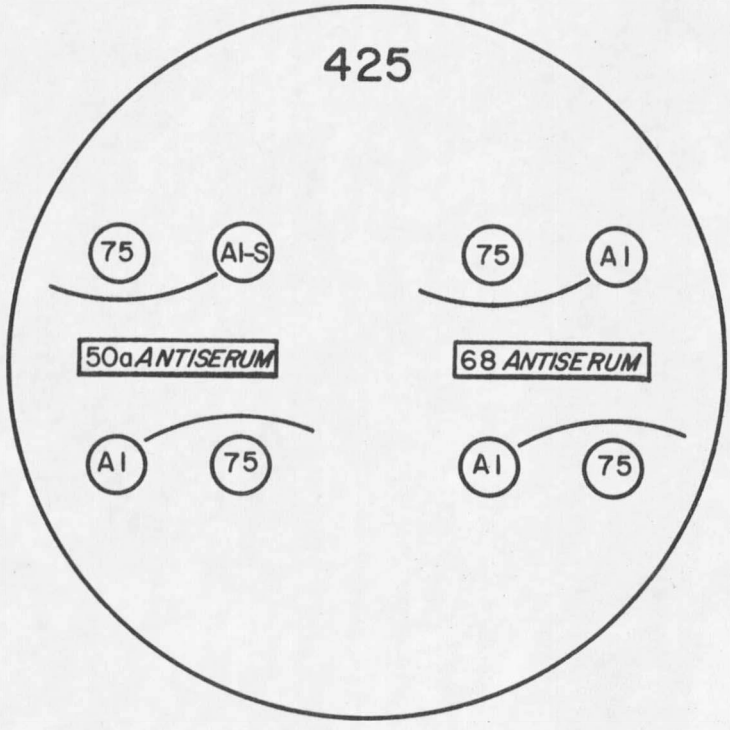


Fig. 12 Adjacent plating of A. spicatum (#75-3) and A. hirsutum (#A1-S). The precipitate lines which the two had in common are not shown. Note the unique precipitate line for A. spicatum. Two sources of antiserum were used, A. panormitanum (#50a-7) and A. sibiricum (#68-5)

reaction which neither of the supposed parent species had (Figure 13).

II. CYTOLOGY AND MORPHOLOGY OF THE CRESTED WHEATGRASSES

The main underlying principle of systematic serology is that the antigens compared are representative of the organisms producing them, in the same sense and for the same general reasons that their corresponding structures are, that is, phenotypic expressions. It is not suggested that serological methods of systematics be used to the exclusion of others, but rather than serology may yield important data which can be used along with data from other fields to give a clearer idea of the relationships of the species being studied. The section Agropyron of the genus Agropyron was selected for a more detailed study in an effort to correlate morphological, cytological, and serological results.

(a) Morphology

As a part of the more detailed investigation of the crested wheatgrasses involving 7 strains of 6 species, the morphological characteristics of the spike were studied in detail. Photographs of the spikes are shown in Figure 14. Scaled drawings of the glumes, lemmas, paleas, and anthers of each taxon are shown in Figure 15. These drawings were made from the lowermost florets when pollen had formed but anthesis had not yet occurred.

Comparison of the glumes, lemmas, paleas, and anthers showed some striking differences. In general, these parts were found to be smallest in the diploid A. cristatiforme (#10-3), and largest in hexa-

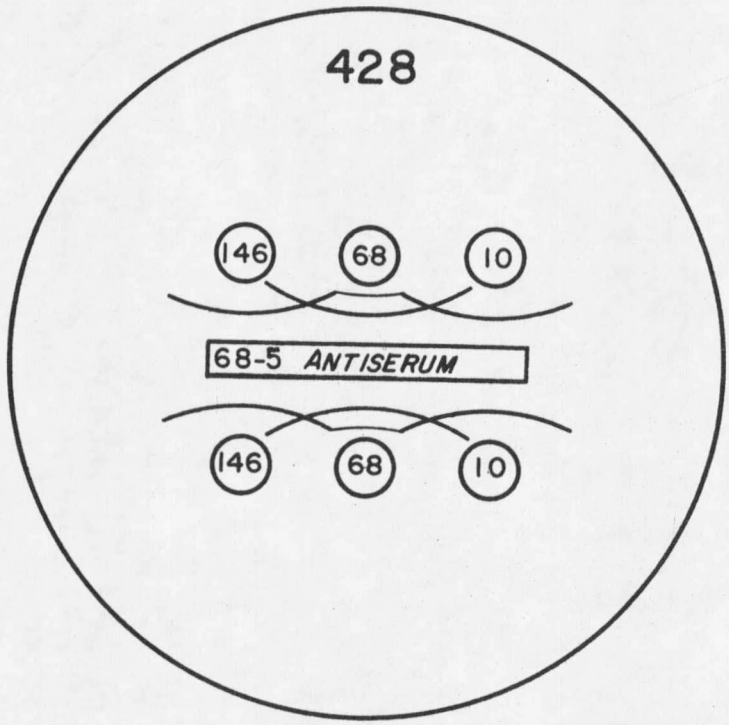


Fig. 13 Testing the hybrid origin of A. sibiricum (#68-5). The supposed parental species are #146-S, A. repens, and #10-3, A. cristatiforme. Note the unique precipitate line of A. sibiricum which indicates that it contains an antigen which the others do not. Common lines are not shown except for one drawn in.

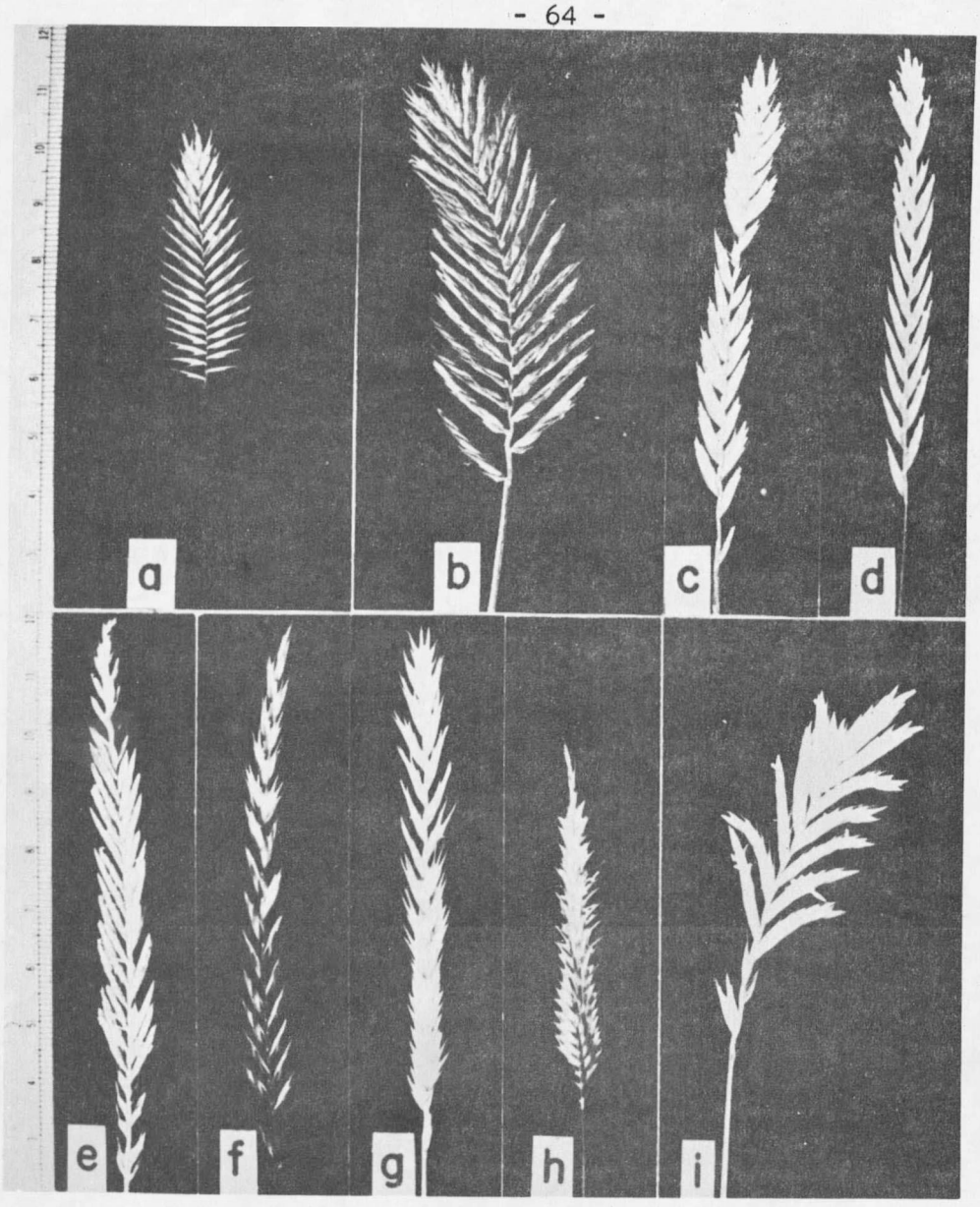


Fig. 14 Spikes of the crested wheatgrasses treated in this investigation; a) A. cristatiforme (#10-3), b) A. imbricatum (#28-9), c and d) A. mongolicum (#48b-1), e) A. sibiricum (#68-5), f and g) A. desertorum (#25-9), h) A. desertorum (#30-9), and i) A. cristatum (#80a-2).

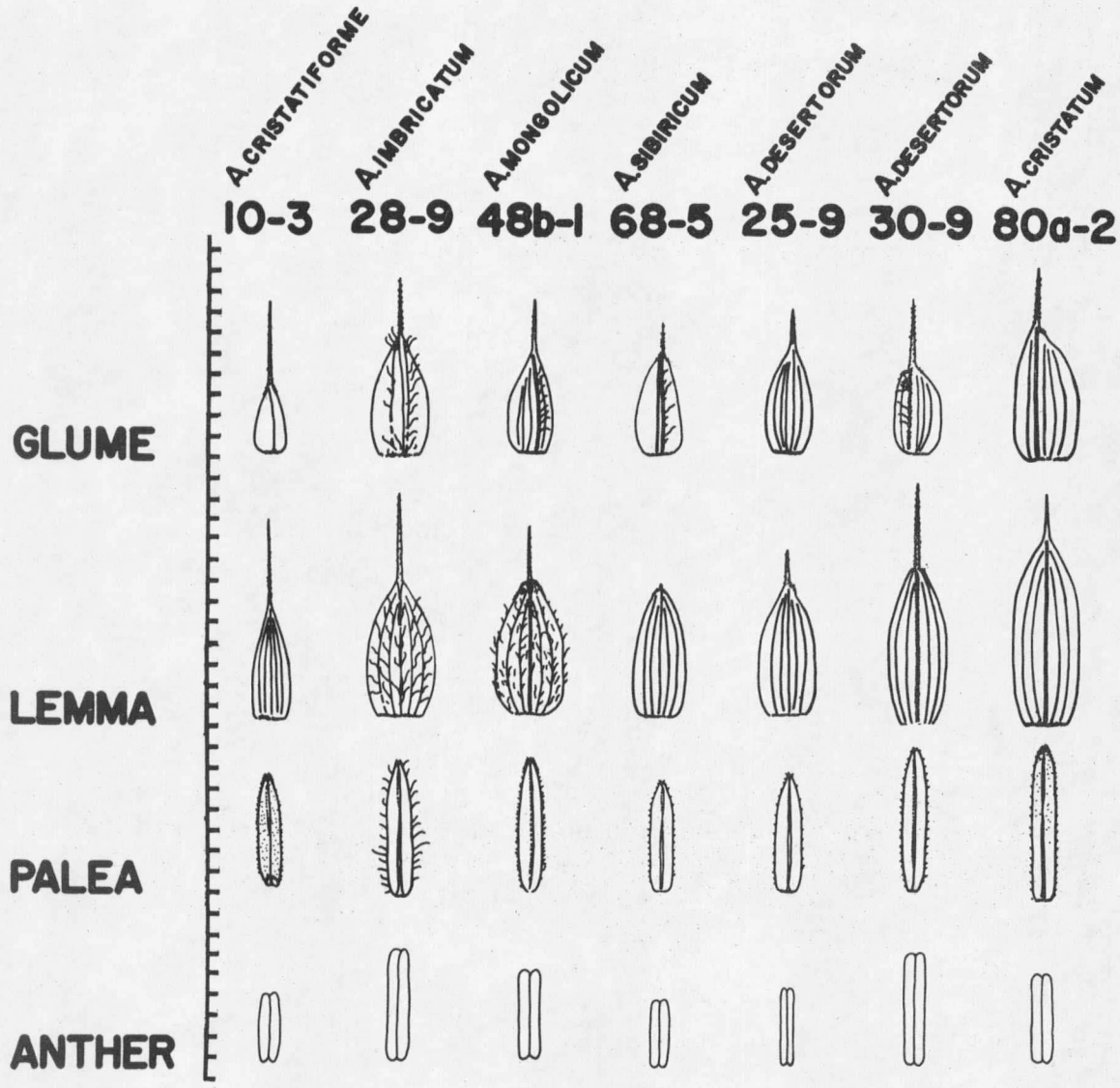


Fig. 15 Scaled drawings of the glume, lemma, palea, and anther of the crested wheatgrasses treated in this investigation. One unit equals 1 mm.

ploid A. cristatum (#80a-2), with the tetraploids somewhat intermediate in size. All glumes, lemmas, and paleas had short hairs, up to 0.1 mm., on the inside arising from short cells. Long hairs, up to 1.5 mm., occurred on the outside of the lemmas of A. imbricatum (#38-9) and A. mongolicum (#48b-1). None of the other forms considered here had long hairs on the outside of the lemma, but A. sibiricum (#68-5) had an abundance of them on the inside near the apex. A. imbricatum (#28-9), A. mongolicum (#48b-1), A. sibiricum (#68-5), and A. desertorum (#30-9) had glumes with long hairs, up to 1.5 mm., but they occurred only along the main rib except in A. imbricatum where the long hairs were well scattered over the whole surface. A. imbricatum (#28-9) was the only species observed that had long hairs on the palea and they occurred only on the two main ribs (Figure 15).

(b) Cytology

Results of a cytological study of the crested wheatgrasses revealed some chromosomal irregularities. Chromosome counts and chromosome associations in pollen mother cells indicated that plant #48b-1, received as A. mongolicum, is an introgressive hybrid. The morphological characteristics are much like those of A. mongolicum. Keng (1938) stated that "although intermediate forms between it and A. cristatum (L.) Gaertn. are also found in this region, the above cited specimens are distinguished from A. cristatum by the relatively longer but narrower spikes and the pubescent or nearly glabrate rachis." Since most of the cells are aneuploid (Table V), it appears reasonable to assume that one

of the parents had a chromosome count in excess of the tetraploid. The chromosome counts were found to range from 2n=28 to 2n=36. Sixty-nine percent of the cells counted had chromosome counts of 2n=32, (Figure 16). Multivalents were common in this plant with 80% having one or more quadrivalents and 40% having a hexavalent. Seventy percent of the cells had univalents, 20% had one or more trivalents, and 10% had a pentavalent (Table VI).

Agropyron imbricatum (#28-9) was found to be an aneuploid plant also. Seventeen chromosome counts ranged from 2n=28 to 2n=34, with 10 counts being 2n=30 (Figure 17b). All chromosomes were associated as bivalents; no univalents or multivalents were observed. Figure 17a shows a cell in late anaphase I in which there is a laggard.

The hexaploid crested wheatgrass identified by Dr. J.R. Swallen as A. cristatum (80a-2) had a fairly regular meiosis. For the most part, the chromosomes were associated as bivalents, but an occasional quadrivalent was found (Figure 18). Dewey (1961a) observed some hexavalent associations in a similar hexaploid strain of A. cristatum.

All other species of the crested wheatgrasses used in this study were normal tetraploids with the exception of strain #10-3 of A. cristatiforme, which was diploid (2n=14). No irregularities were observed in the meiosis of A. cristatiforme. Chromosome counts of the other species were made from root tip squashes; their meiosis was not studied.

Table V. Chromosome counts from 42 cells of A. mongolicum, plant 48b-1

2n number----------	28	29	30	31	32	33	34	35	36
No. of cells	2	0	3	1	29	0	4	0	3
Percentage	4.7	0	7.1	2.4	69.0	0	9.4	0	7.1

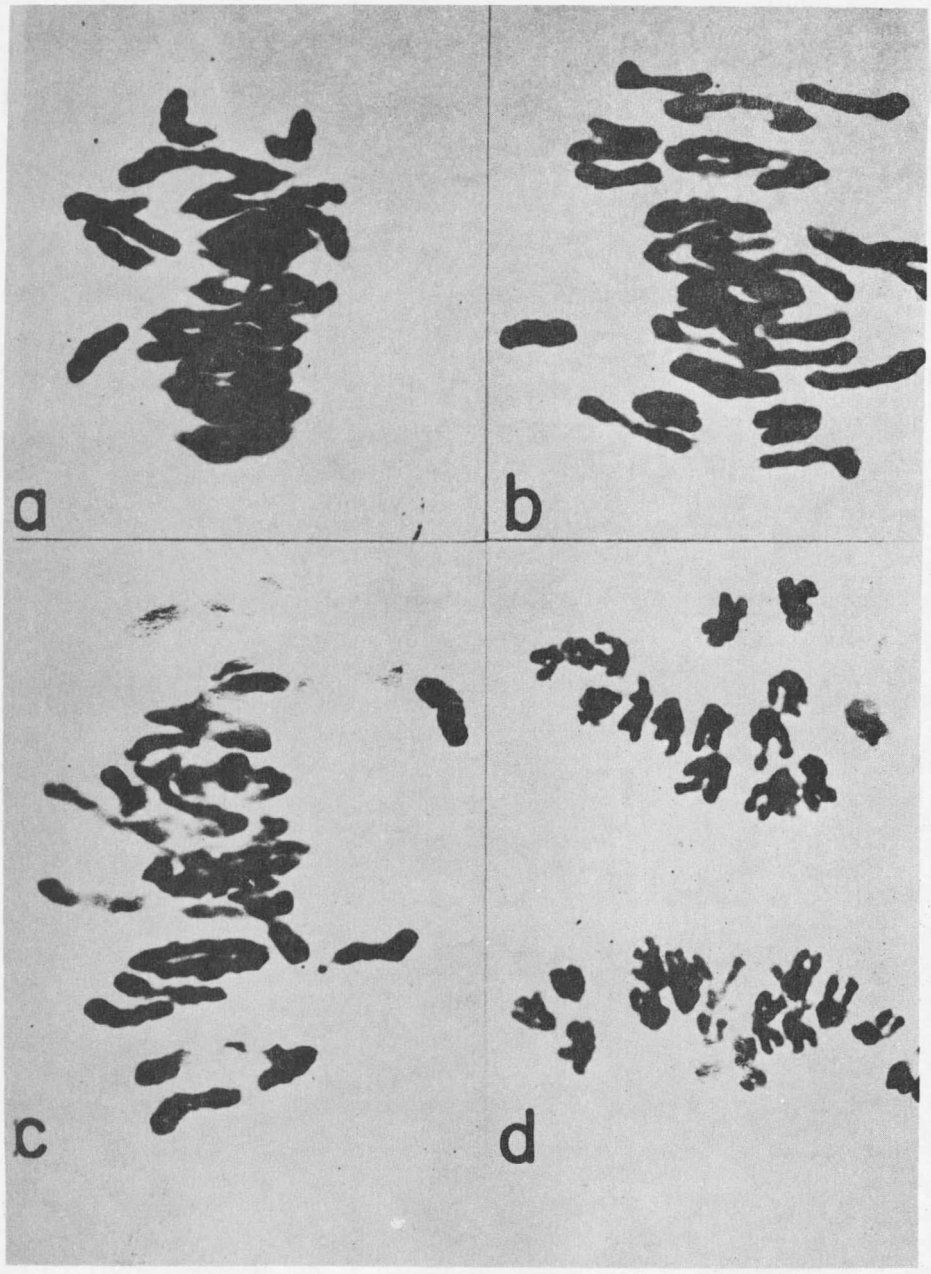


Fig. 16 Cytology of Agropyron mongolicum; a, b, and c show multivalent association of chromosomes; d, shows the aneuploid nature of the plant.

Table VI. Chromosome associations at metaphase I in 10 pollen mother cells of A. mongolicum, plant #48b-1.

2n No.	No. Of PMC'S	Number of observed associations					
		I	II	III	IV	V	VI
28	1	4	8	0	2	0	0
32	2	0	9	0	2	0	1
32	1	2	13	0	1	0	0
32	1	6	7	0	3	0	0
32	1	0	12	0	2	0	0
32	1	2	11	1	0	1	0
34	1	2	9	0	2	0	1
34	1	2	11	0	1	0	1
34	1	8	7	4	0	0	0

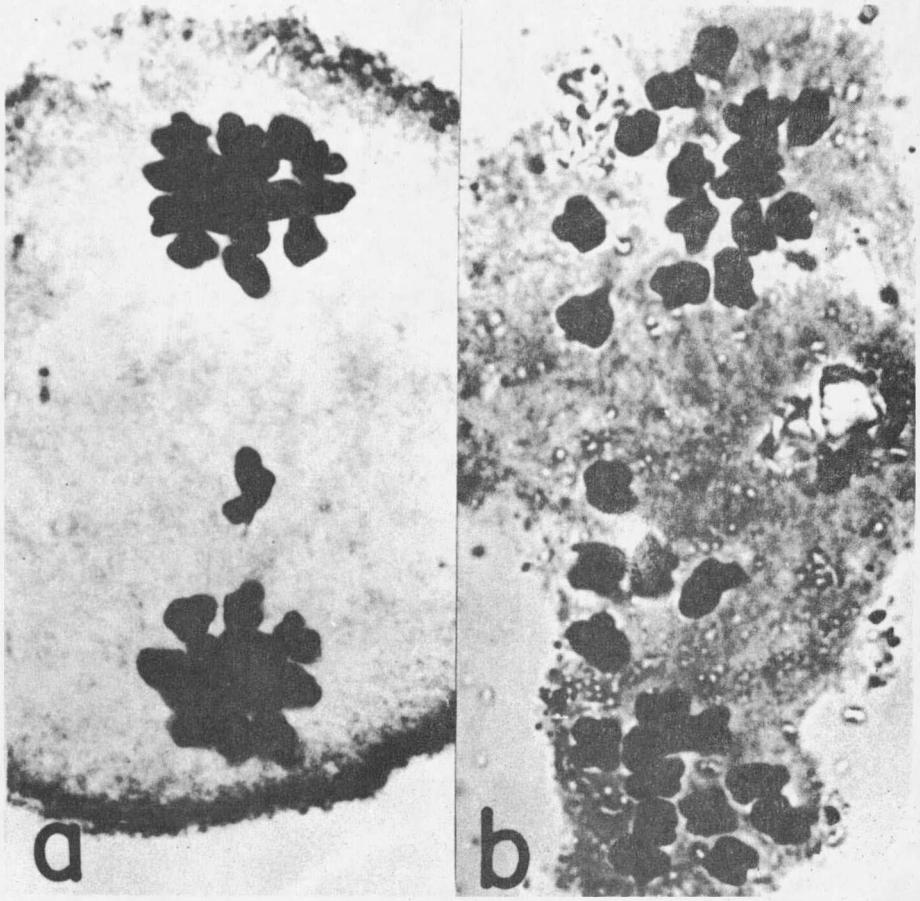


Fig. 17 Meiotic chromosomes of A. imbricatum; a) laggard and b) chromosomes of late anaphase I.



Fig. 18 A pollen mother cell of A. cristatum (#80a-2) showing a quadrivalent association of chromosomes.

DISCUSSION

I. PRELIMINARY REMARKS

When working with a large number of species to determine their serological relationships, it appears impractical to produce antiserum to extracts of each species. During the present study it seemed best to select a few species to induce antibody production. At the inception of this investigation it was not known whether the 4 sections of the genus Agropyron (Goulardia, Holopyron, Agropyron, and Eremopyrum) were valid. Upon qualified acceptance of the sections, a single species was selected from each, and its antigens used to stimulate production of antibodies (Table II).

Each species tested probably has proteins which are identical with some of those of the species used to stimulate antibody production and some which are different. Many of the proteins (antigens) of these species may be basic and common to most grasses while additional ones may be shared by subgroups. Beyond this a species would be expected to have unique proteins or combinations of proteins making it different.

The Four-way test was designed especially for this work with grasses of the genus Agropyron, but may have wide application. The test can be used to identify species which have a close serological relationship. The Four-way test is not one of absolute accuracy---it only points out minimum differences.

There are, in fact, at least two ways in which error may enter

the data. One source of error is concerned with the number of precipitate lines counted. For example, if species A reacting with #10-3 antiserum (A. cristatiforme) forms 7 lines while species B reacting with the same antiserum produces 10 lines, they have a calculated difference of 3 lines. This is the minimum difference possible, but the difference may be greater. The greatest number of lines possible with reactants of this antiserum is 17, the number which was produced by reaction with A. cristatiforme (#10-3). If species A had 7 lines while species B had 10, it is conceivable that none of these would be common to the two species. If species C and D produced 16 lines each in reaction to the antiserum, the calculated minimum difference would be zero when, in fact, the difference could be 2--the maximum difference. The second source of error is in counting and was explained in the methods. These sources of error do not lessen the usefulness of the Four-way test, provided its limitations are recognized.

In this Four-way test, the serological difference revealed is minimal. If the minimum serological difference is large as indicated by the difference index, there appears to be no need to study the relationship in detail. If, however, the difference index indicates a possible close serological relationship, the degree of relationship can be investigated by other means such as the antigen absorption test or adjacent plating. The use of such means in the present investigation has revealed that the Four-way test is re-

liable.

II. SEROLOGY, CYTOLOGY, AND MORPHOLOGY

The serological results of this study indicate a scheme of relationships between species (and strains) of the genus Agropyron which is, in some instances, similar to that already postulated on the basis of morphological and cytological studies. Dewey (1961b) studied natural and controlled hybrids of A. repens (2n=42) and A. desertorum (2n=28) and found that the hybrid had a chromosome count of 2n=35 as expected. He stated "The hybrid plants were esentially sterile...". As a result of his cytological study of the parental plants and the hybrid, he concluded that A. repens and A. desertorum have no genomes in common. The genome formula of AAAA was determined for A. desertorum and B_1 B_1 B_2 B_2CC for A. repens. When these two species were compared in the Four-way test, they had a difference index of 0.20 indicating that they are serologically distantly related---a finding which agrees with cytological evidence.

Dewey (1963a) concluded from his study of a synthetic hybrid, A. trichophorum X A. desertorum, that the parental species share 1 or 2 partially homologous genomes. The genome formula assigned for A. trichophorum was $A_1A_1B_1B_1B_2B_2$. The genome formula of A. desertorum is stated above. It is to be expected that the difference index would be lower in species that have some homologous or partially homologous genomes as is the case with these species.

Their difference index of 0.12 indicates a close relationship, which agrees with cytological data reported by Dewey.

Dewey (1963a) also reported that A. trichophorum and A. intermedium bear the same genomes. Based on this finding, a low difference index would be expected. The observed difference index was 0.10 which showed a close serological relationship.

Stebbens and Pun (1953) assigned a genome formula of $E_1E_1E_2E_2NN$ to A. intermedium, a formula with which Cauderon (1958) agreed when she reported that the E genomes are modifications of the A. elongatum genome (these E genomes are probably equivalent to the A genomes proposed by Peto, 1936, and Dewey 1963a). If this is correct, a low difference index of a comparison of A. intermedium and A. elongatum would be expected. When A. elongatum (#36-5) was compared with A. intermedium (#44-6), the difference index was 0.08, indicating a close serological relationship. When the other two strains of A. elongatum, (#35a-2, diploid and #37-5, hexaploid) were compared with A. intermedium, difference indexes of 0.14 and 0.10, respectively, were observed.

If the genomes of A. trichophorum and A. intermedium ($A_1A_1B_1B_1B_2B_2$) are the same as reported by Dewey (1963a), the Four-way test comparison of A. trichophorum with the 3 ploidy levels of A. elongatum should result in low difference indexes, as in the comparison of A. intermedium and A. elongatum. The difference indexes in the comparison of A. trichophorum (#104-3) with A.

elongatum, strains #35a-2 (AA), #36-5 (AAXXXXYYYY) (Peto 1936), and #37-5 were 0.12, 0.16, and 0, respectively. These results may indicate that the genomes of A. trichophorum (#104-3, hexaploid) and A. elongatum (#37-5, hexaploid) are the same, and that there is a homology or partial homology with some of the genomes of the other strains of A. elongatum.

Synthetic hybrids of A. trichophorum and hexaploid A. cristatum were studied morphologically and cytologically by Dewey (1963c). He concluded, on the basis of chromosome association in pollen mother cells in the parent species and their hybrids, that one of the A. trichophorum genomes was partially homologous with the genomes of hexaploid A. cristatum. The genome formulae which he determined are as follows: A. cristatum AAAAAA, A. trichophorum $A_1A_1B_1B_1B_2B_2$, and their hybrid $AAAA_1B_1B_2$. Considering the partial homology of genomes of the two parent species, a relatively low difference index would be expected when they were compared in the Four-way test; a difference index of 0.12 was observed.

Based on cytological and chromotographic studies Schulz-Schaeffer, et al. (1963a) proposed a genome formula of A_1A_1 for A. cristatiforme Sarkar, $A_1A_1A_2A_2$ for A. desertorum, and $A_1A_1A_1A_1A_2A_2$ for A. cristatum (hexaploid strain). A. desertorum (#s 25-9 and 30-9) and A. cristatum (#80a-2) had a difference index of zero which suggests that the three had the same genomes. The results of the Four-way test lend support to the genome designations proposed.

Since the polyploids A. desertorum (#s 25-9 and 30-9) and A. cristatum (#80a-2) contain some genomes not identical with those of A. cristatiforme, a difference index greater than zero would be expected. The observed difference indexes were 0.08 for the three comparisons, confirming that the A_1 and A_2 genomes show a certain degree of difference which is indicated by the subscripts of the genome symbols.

Cauderon (1962) proposed a genome formula of $J_1J_1J_2J_2E_3E_3$ for A. junceum subsp. mediteraneum and $E_1E_1E_2E_2N_1N_1$ for A. intermedium indicating that the two species have some genomes which are partially homologous. A relatively low difference index would be expected. Data of the Four-way test show that a comparison of A. intermedium (#44-6) and A. junceum subsp. mediterraneum (#140-3) resulted in a difference index of 0.14 which is about as expected.

Stebbins (1949) attempted to hybridize A. trachycaulum and A. dasystachyum but was not successful. In view of the fact that they could not be hybridized, a high difference index would be expected. When they were compared in the Four-way test, a difference index of 0.20 was found.

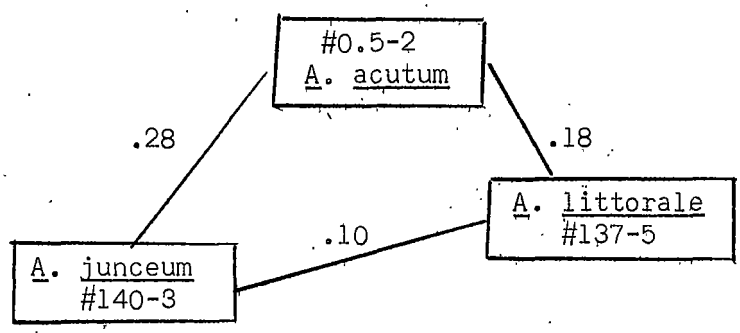
Stebbins and Snyder (1956) reported a hybrid between A. trachycaulum, section Goulardia, and A. spicatum, section Holopyron. They emasculated and artifically pollinated 57 florets from which they harvested 12 seeds. From these 12 seeds they grew 10 F_1 plants. A close serological relationship would be possible and data from

Table III show a difference index of 0.12.

Godley (1947) assigned a genome formula of $A_1A_1A_2A_2BB$ to A. pungens and $A_1A_1A_{2a}A_{2a}CC$ to A. repens. Based on the homology and partial homology of some of the genomes of the two species a low difference index should be found. Data from Table III show a difference index of 0.08.

Sakamoto (1957) studied natural and artificial hybrids between A. tsukushiense (2n=42) and A. mayebaranum (2n=42). He reported 21 bivalents in a meiotic study of the hybrid and stated "Their complete sterility may be due to cryptic structural differences between the chromosomes of the two species." Their difference index was 0.04, corroborating Sakamoto's conclusion regarding the cause of sterility.

Cauderon (1958) reported that A. acutum is a natural interspecific hybrid resulting from a cross of A. junceum subsp. mediterraneum (2n=42) and a hexaploid Agropyron species, probably A. littorale. The difference indexes are as follows:



These differences indicate that A. acutum (or at least this one plant) was not a hybrid of the two supposed species (but this accession 0.5 did come from a hybrid population). The Montana State College accession #0.5-2 received as A. acutum is fertile in contrast to the A. acutum reported by Cauderon which is sterile.

The results of the present investigation were not in complete accord with the cytological results reported by Schulz-Schaeffer and Jurasits (1962) who made a cytological study of karyotypes of 25 species of the genus Agropyron and typified each by their satellite chromosomes. They reported that "Fifteen of the 25 investigated species have a pair of satellite chromosomes of type F-1 or F-2..... This type seems to be the indicator chromosome for the Agropyron genome A_1A_1 which Stebbins and Pun (1953) thought to be widespread among diploid species and a component of many polyploid species of Agropyron...." This type satellite chromosome did not appear in the 3 species they worked with from the section Agropyron.

If the F-1 and F-2 types of satellite chromosomes are indicators of the A_1A_1 genome, a relatively low difference index would be expected when species containing this type of genome are compared, especially if they are diploid or tetraploid. Schulz-Schaeffer and Jurasits (1962) found that A. latiglume (2n=28) and A. panormitanum (2n=28), both of the section Goulardia, had the F-1 type. The serological comparison of these two species showed

a difference index of 0.20 which is greater than expected, but may reflect differences of their other genomes. Difference indexes of 0.12 and 0.14 were observed when A. latiglume was compared with 5 species (section Holopyron) which had a satellite chromosome of type F-1. The three species which they studied from the section Agropyron had no satellite chromosomes which they could detect and were different in this respect from species of other sections with the exception of A. triticeum, section Eremopyrum. This agrees with the serological results of this study.

Generally speaking, the relationships of the species of the genus Agropyron are fairly consistent in their serological values with the cytological relationships others have observed. However, the tests used here revealed some unexpected results inconsistent with the traditional systematic classification.

In three cases the Four-way test showed that two different species may be serologically identical (difference index of zero). For example, A. brachyphyllum and A. donianum both produced 12 lines in reaction with A. cristatiforme antiserum when 17 was the maximum possible. At least 7 of the antigens must be common to the two species, and it could be expected that probably more were in common. In reaction to the other three antisera used in the Four-way test they produced the same number of lines, 8 in each case. In reaction with A. triticeum antiserum where a maximum of 12 lines was possible, each produced 8, at least four of which must have been in

common. The reaction with A. dasystachyum antiserum in which 9 lines were possible, showed that the two species had at least 7 antigens in common. The reaction with A. panormitanum antiserum is analogous to that of A. triticeum antiserum.

The observation that two different species produced the same number of precipitate lines in reaction to four different antisera seemed to warrant further investigation to determine the true, rather than the minimum, serological difference. A new and different antiserum was produced in response to A. sibiricum antigens for use in this test and therefore was not specific for either of these two species being tested. If absorption was complete, no lines should appear by the other if it has proteins in common with A. sibiricum (the antibody stimulant) which A. brachyphyllum does not have. Neither formed lines; that is, A. donianum had no antigens in common with A. sibiricum which A. brachyphyllum did not also have. But this test did not eliminate the possibility that A. brachyphyllum might have antigens in common with A. sibiricum which A. donianum does not have.

With this possibility in mind, the reciprocal absorption test was made; that is, the antiserum was absorbed with antigens of A. donianum. The results were identical with the first absorption test, thus establishing that A. brachyphyllum and A. donianum have the same antigens in common with A. sibiricum. It must be pointed out, however, that if antiserum was produced to either A. brachy-

phyllum or A. donianum and absorption tests made, that some differences probably would be found.

Another comparison of two species, A. elongatum (#37-5) and A. trichophorum (#104-3), revealed that they may be serologically identical (difference index of zero). They had serological reactions very much like A. donianum and A. brachyphyllum. A. donianum and A. brachyphyllum each produced one less line in reaction with A. cristatiforme antiserum than did A. elongatum (#37-5) and A. trichophorum (#104-3). Other reactions resulted in the same number of lines for the 4 species.

On the assumption that they were closely related serologically, the relationships of these four species were tested by absorption. When A. brachyphyllum, A. donianum, A. elongatum (#37-5), and A. trichophorum (#104-3) were allowed to react with unabsorbed antiserum of A. sibiricum, all four produced lines common to all the others and the lines joined by smooth curves. If antigens of two species plated side by side are allowed to react with the same antiserum, all lines which join by a smooth curve and without a spur are considered to be the results of identical reactants. No spurs or unique lines were observed in this case.

To further test the probable close relationship of these four species, their antigens were allowed to react with antiserum which had been absorbed with antigens of one of the four. This was repeated by absorbing the antiserum with different antigens each

time. The antigens of each species completely removed all those antibodies from the antiserum with which the other three might react. All four species had identical reactions with antiserum produced to A. sibiricum, definitely establishing their close serological relationship. It is of interest to note that A. brachyphyllum and A. donianum belong to the section Goulardia while the other two belong to the section Holopyron. Both sections are very closely related morphologically and such a classification of species may seem artificial.

The other instance in which the difference index of two species was found to be zero in the Four-way test concerns A. desertorum and hexaploid A. cristatum. The interpretation of their serological relationship will be given later in the analysis of the crested wheatgrasses studied.

It is to be emphasized that this work was not primarily concerned with intraspecific variation. In most cases a single plant of a species was used and this plant may not be the most representative. Incidental to the main purpose of this study some information was gained relative to intraspecific variation. For example, the two strains of A. trichophorum, #57-5 and #104-3, had a difference index of 0.10 which is a greater difference than many other species had when compared to each of these strains. The two strains of A. trichophorum were from different sources as noted in Table i. Strain #57-5 was introduced as A. popovii. Another

example of testing two strains of the same species is concerned with A. desertorum, #25-9 and #30-9. These were found to be serologically identical by the Four-way test and the absorption test. Both sources of seed originated in Russia. The third example is concerned with three strains of A. elongatum discussed below.

A comparison of difference indexes in the polyploid series of A. elongatum revealed some surprising relationships. It was supposed that they would show a close serological relationship since they are considered to be the same species. But the three strains of A. elongatum, #35a-2 (diploid), #37-5 (hexaploid), and #36-5 (decaploid), were not as closely related serologically as supposed. Peto (1936) proposed a genome formula of AAXXXXYYYY for the decaploid strain which he studied and AA for the diploid (the AA genomes used by Peto are probably the same as the EE genomes proposed by Stebbins and Pun, 1953, and Cauderon, 1958). Peto has shown that the decaploid has 8 genomes which differ from those of the diploid and it could thus be very different. It would be more closely related to a species of a genome formula of XXYY than to one of AA. The Four-way test showed that the hexaploid and decaploid strains were more closely related than either was to the diploid. The morphology of the two polyploids is more similar than that of either compared with the diploid. This is consistent with the findings of the Four-way test.

Each of the strains was more closely related to other species

than to other strains within the species. The diploid (#35a-2) was more closely related to 7 other species than to polyploid strains of A. elongatum. Approximately 1/3 of the species tested, 16 of the 41, had closer relationships to #36-5 (decaploid) than did the other two strains of the same species. Even though the two polyploids were more closely related to each other than to the diploid, A. brachyphyllum, A. donianum, A. trichophorum (#57-5), and A. semicostatum were all more closely related to #36-5 (decaploid) than was #37-5 (hexaploid).

The relationship of hexaploid A. elongatum (#37-5) and A. trichophorum (#104-3) was of interest because they were serologically identical in reaction in the Four-way test and in the antigen absorption test. Because antiserum was not produced to either of the species, it cannot be said that they were identical with respect to all their antigens. They were, however, much more closely related than the hexaploid A. elongatum was to either of the other two strains of its species. Approximately 1/2 of the species tested were more closely related to the hexaploid (#37-5) than the diploid (#35a-2) was and 4 were more closely related to the hexaploid (#37-5) than the decaploid (#36-5) was.

According to the data from the Four-way test A. brachyphyllum (#3-2) and A. donianum (#32-1) were related to strain #37-5 by a difference index of 0.02, but in the absorption tests they had identical reactions with the antisera used in this study.

From the data of this investigation it seems that the three strains of A. elongatum tested might be best treated as separate species. The evolution of the polyploids of A. elongatum appears to warrant much more study.

The two strains of A. desertorum were serologically identical. The crested wheatgrasses, A. desertorum (strain #s 25-9 and 30-9, both tetraploid) and A. cristatum (#80a-2, hexaploid), had difference indexes of zero when compared in the Four-way test. A further test with the adjacent plating technique, in which extracts of the 3 reacted with A. sibiricum antiserum, revealed that they formed the same precipitate lines, indicating identical antigens.

Serological comparison of 7 strains representing 6 species of crested wheatgrass indicated some striking differences. When compared with A. cristatiforme (#10-3) in the absorption test, A. mongolicum (#48b-1) and A. imbricatum (#28-9) differed by a single precipitate line (Figure 19), although the lines were different. This indicates that these two species are more closely related to A. cristatiforme than the other species tested. A. sibiricum (#68-5) differed from A. cristatiforme by two precipitate lines. These two lines are the same as the ones mentioned above for A. mongolicum and A. imbricatum. The two strains of A. desertorum (#25-9 and #30-9) and A. cristatum (#80a-2) differed from A. cristatiforme by 3 precipitate lines. These 3 species were found to be serologically identical when compared to A. cristatiforme.

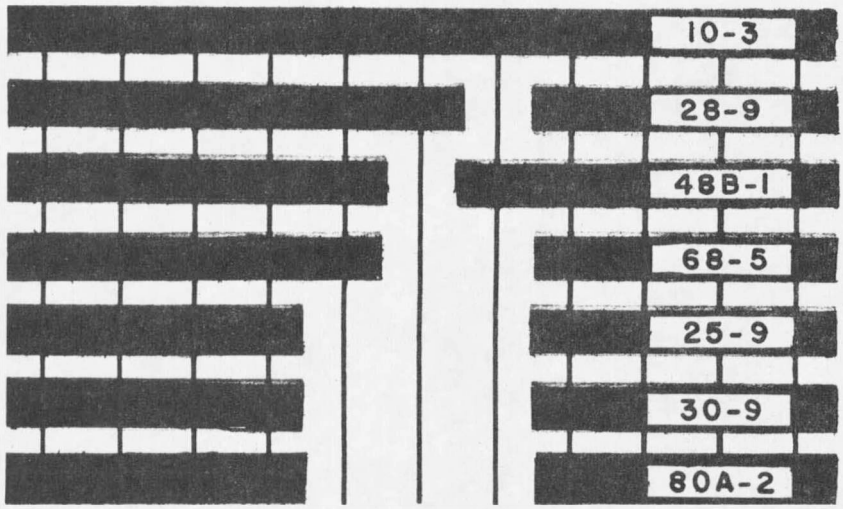


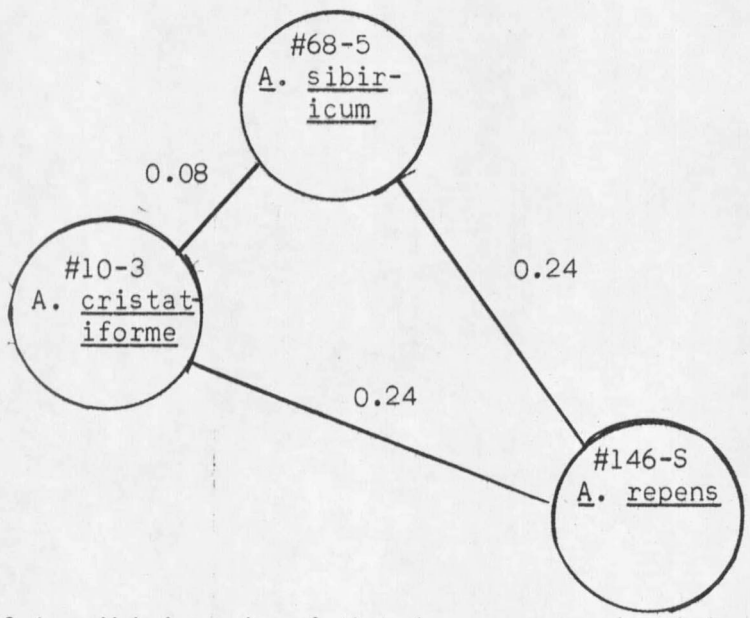
Fig. 19 Comparison of 7 strains of 6 species of crested wheatgrass; 10-3, A. cristatiforme; 28-9, A. imbricatum; 48b-1, A. mongolicum; 68-5, A. sibiricum; 25-9 and 30-9, A. desertorum; and 80a-2, A. cristatum. Horizontal bars overlap the vertical lines (representing antigens) which each species has in common with the others.

From these data it appears that A. mongolicum (#48b-1) and A. imbricatum (#28-9) are more closely related to A. cristatiforme than are A. sibiricum (#68-5), the two strains of A. desertorum (#s 25-9 and 30-9), and A. cristatum (#80a-2). A. sibiricum (#68-5), in this serological comparison, appeared to be more closely related to A. cristatiforme than the two strains of A. desertorum (#s 25-9 and 30-9) and A. cristatum (#80a-2).

Serology obviously does not settle whether the crested wheatgrasses treated here are different species or are merely different forms of the same species. If one considers, as many do, that A. sibiricum is a valid species, then one might also conclude that the two ploidy forms often referred to as A. cristatum (the diploid A. cristatiforme Sarkar and hexaploid A. cristatum of this study) are distinct species because this work shows that A. sibiricum is more closely related to A. cristatiforme than is the hexaploid A. cristatum. It must be pointed out, however, that the polyploids compared to diploid A. cristatiforme may have other genomes which might make them differ more than is indicated by this study.

One additional source of antiserum was produced in response to a tetraploid crested wheatgrass, A. sibiricum (#68-5), to test the proposals of Troitskii (1932) and Tronickij (1959) that A. sibiricum is of hybrid origin resulting from a cross of A. repens and A. cristatiforme. They found, in the same area where the supposed parents species grow, some plants which fit the description of A.

sibiricum as well as other intermediate forms. If the genome formula for A. cristatiforme, A_1A_1 (proposed by Schulz-Schaeffer, et al., 1963a), and that for A. repens, $B_1B_1B_2B_2CC$ (proposed by Dewey, 1961) are correct, the hybrid should have genomes $A_1B_1B_2C$ and probably would be sterile. In contrast, A. sibiricum is fertile. The Four-way test shows relative serological relationships as determined by difference index numbers as follows--



If A. sibiricum is of the above proposed origin, and therefore has 3 times as many genomes from A. repens as from A. cristatiforme, it would be reasonable to assume that it would have a closer serological relationship to the contributor of the 3 genomes. Evidence from the Four-way test seems to refute the supposed hybrid origin of A. sibiricum. Additional evidence was derived from the adjacent plating method. It showed that A. sibiricum had one antigen which neither of the supposed parent species had. However, "hybrid

antigens" have been demonstrated in synthetic hybrids of undisputed parentage (Irwin, 1951; McGibbon, 1944). Both the presence of this unique antigen and the large difference indexes of the Four-way test rather strongly argue against the suggested hybrid origin of A. sibiricum.

The systematic classification of the crested wheatgrasses has traditionally shown that they are distinct from the other Agropyron species. Several groups of species studied here other than the crested wheatgrasses emerged as clusters characterized by serological affinities and may demonstrate recent speciation or evolution. Several of these groups are recognizable in the three dimensional model (Figure 20). One such cluster involved A. elongatiforme, A. spicatum, and A. hirsutum. A spicatum occupied a central position and differed from the other two by a difference index of 0.02. A. elongatiforme and A. hirsutum had a difference index of 0.04. The morphology of the three species in this group is radically different, but was not studied in any detail. A second conspicuous group or cluster is that represented by A. tsukushiense, A. mayebaranum, A. scabrifolium, and A. pycnanthum which were serologically quite closely related and represent, in all probability, the most aberrant cluster.

One of the most remarkable and least expected close serological relationships revealed by this investigation, as mentioned above, was that of the old world annual A. hirsutum (#A1-S), section

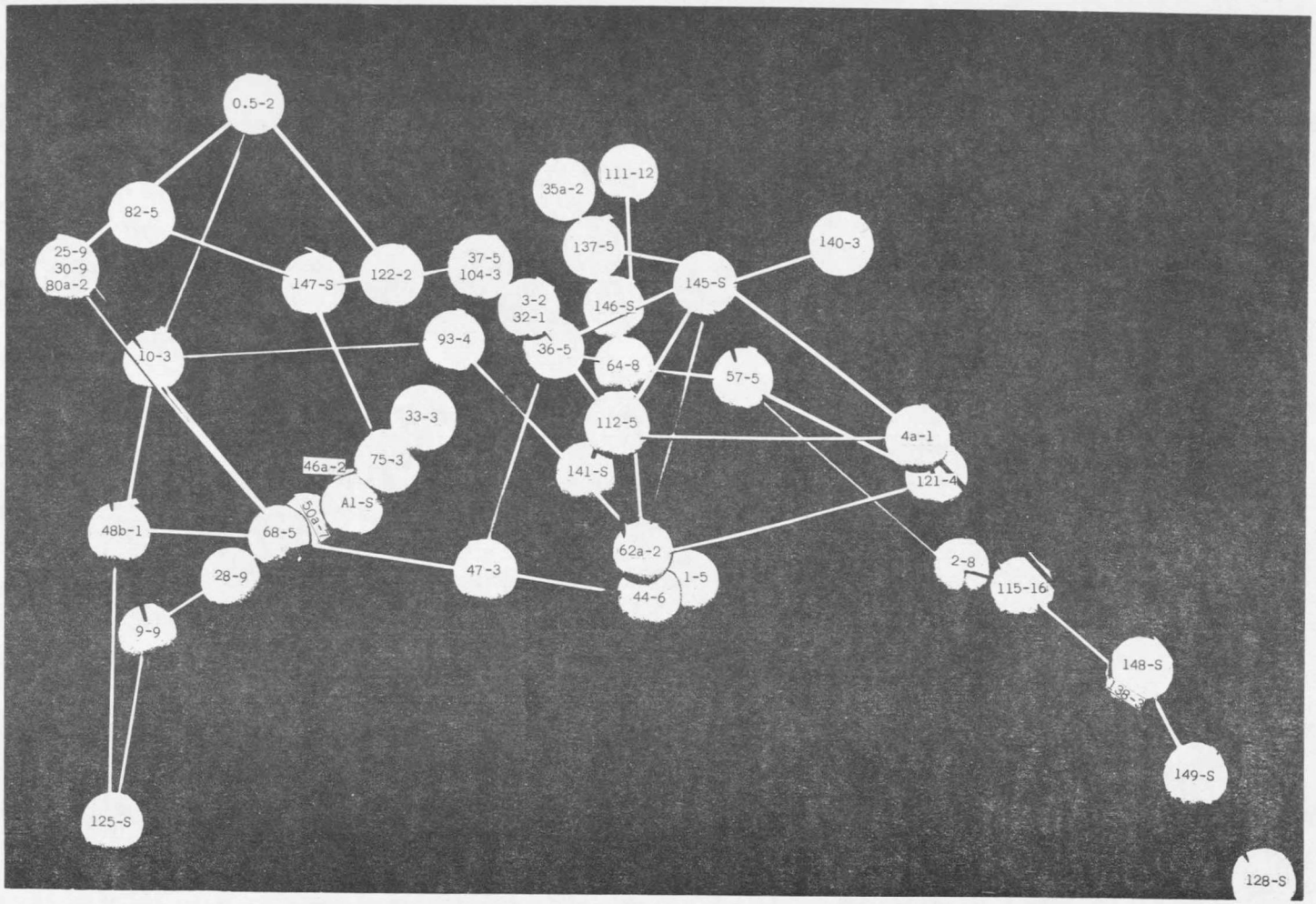


Fig. 20 Three dimensional model showing the serological relationships of 45 strains of 41 species of the genus Agropyron.

Eremopyrum, and the new world perennial A. spicatum (#75-3), section Holopyron. The Four-way test revealed a difference index of 0.02 when these two were compared. They differed only in the number of precipitate lines produced in reaction with A. panormitanum antiserum; A. spicatum produced one more line than did A. hirsutum. Because the Four-way test reveals only minimum differences, it might be suspected that the true serological difference was somewhat greater. In the adjacent plating test using A. sibiricum antiserum, A. spicatum produced one more precipitate line than did A. hirsutum. All the antigens which A. hirsutum had in common with A. sibiricum were also found in A. spicatum. Testing with five different sources of antiserum confirmed that A. hirsutum and A. spicatum have a very close serological relationship.

The fact that A. hirsutum and A. spicatum show a close serological relationship would seem justification for an attempt to hybridize the two species, but this was not done as a part of this study. A search of the literature did not reveal any such attempts. Plans are made to try this cross in the summer of 1964.

Only two annual species of the wheatgrasses (section on Eremopyrum) were treated in this work, A. hirsutum and A. triticeum. The two annuals had a difference index of 0.10 which indicates a close relationship. Unfortunately only a very small amount of extract from A. hirsutum was available, thus ruling out possible absorption of antisera with it. When A. triticeum and A. hirsutum

were plated side by side and allowed to react with antiserum produced to A. triticeum, A. triticeum produced two more lines than A. hirsutum, but all lines of A. hirsutum were in common with those of A. triticeum. When A. hirsutum reacted with A. panormitanum antiserum, 8 precipitate lines formed while A. triticeum formed only 7 lines. This clearly demonstrates that the two annual species each contained specific antigens.

III. GENERAL DISCUSSION

A serological investigation has not usually been carried out with such a large number of species. Ordinarily only a few were selected because of special interest and/or a compounded difficulty in presenting data. It is therefore difficult in this study to summarize the serological relationships of the species of the genus in any organizable manner. Leone (1947) and Cather (1955) have used models to demonstrate serological relationships of some animal species. If more than 3 species are tested, the results of their relationship cannot be pictured in two dimensions and must be shown in three dimensions for approximate interpretation. For this reason and because the data of Table III (differences indexes) are quantitative a three dimensional model was constructed to show the relative relationships of all the species tested in this investigation (Figure 20). The relative distance between two balls, each representing a species, indicates their serological relationships. The model is not intended to show the phylogenetic origin or evolu-

tionary path of the species, but rather to associate them as they exist today.

In some cases the picture of the large model (Figure 20) does not adequately show the serological relationship because of the point from which the picture was taken. Consequently some species that were grouped together in the large model were grouped into a small one which could be shown from a more advantageous point of observation.

The fit of the species, that is their three dimensional relationship, in the model was extremely good. Only three of the species, A. fibrosum (#112-5), A. arizonicum (#2-8), and A. ciliatiflorum (#9-9), did not fit well, but were placed according to their best fit. It is speculated that these species did not fit well into the model because their true serological relationships were not revealed in the Four-way test. It should be remembered that the Four-way test indicates minimum differences, and in fact the difference may be greater. In spite of these three misfits the goodness of the fit of all the other species in the model, which was very good, may be interpreted as indicative of the accuracy of the Four-way test.

In addition to the three-dimensional model a two-dimensional plot of the species was made with a separate set of data, showing the serological relationships of each species used in this investigation (Figure 21). The data of the plot agree essentially with

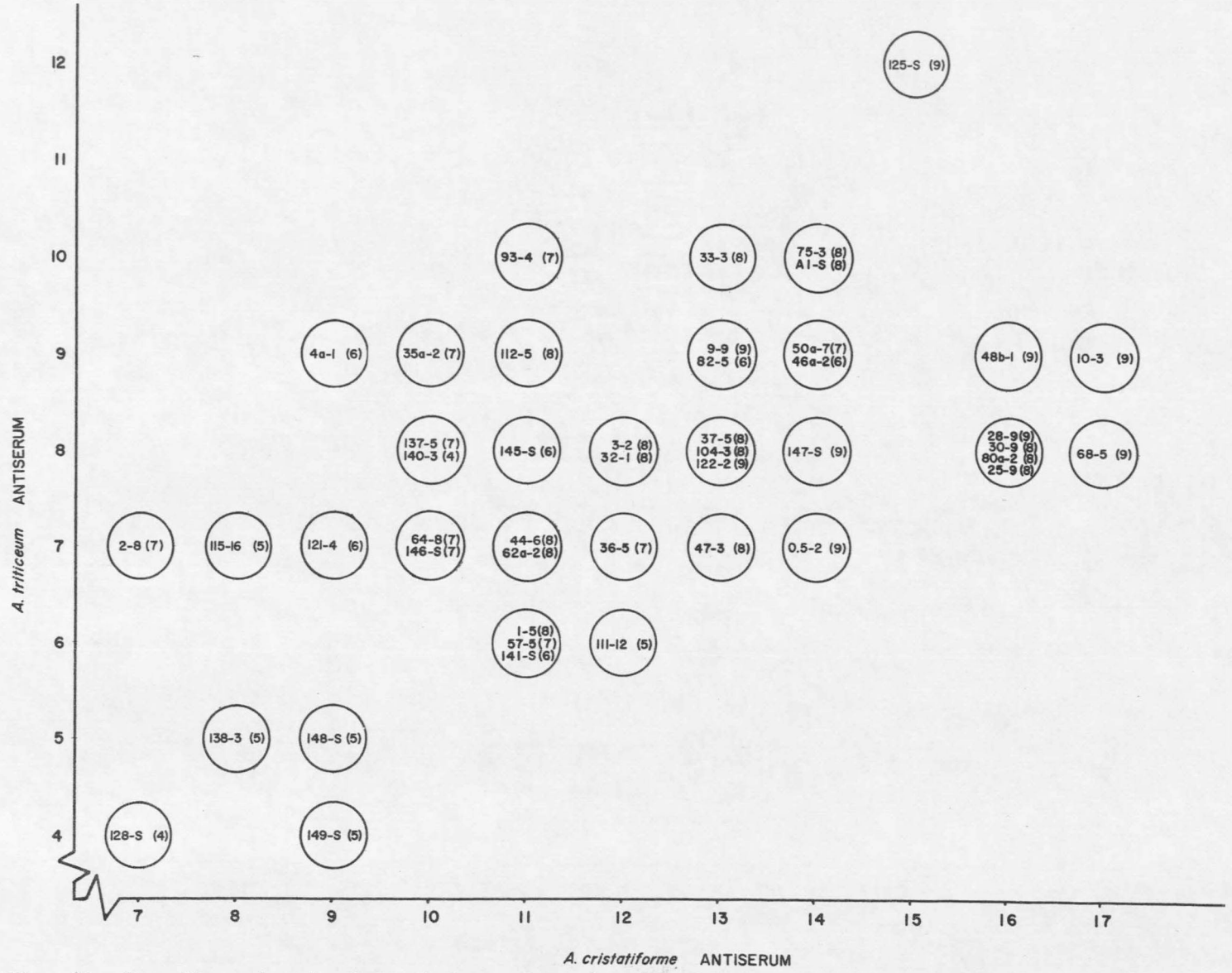


Fig. 21 Two dimensional plot of the serological relationships of some Agropyron species

the relationships shown in the three dimensional model.

It was hoped that, after a serological appraisal of about 1/3 of the species of the genus, a natural classification would be revealed. Nevski (1934) regarded the group of grasses treated in this investigation as belonging to several genera but Holmberg (1926) had already divided the group into four sections of the same genus.

Lorenz and Schulz-Schaeffer (1964) found some correlation with the sections proposed by Holmberg (1926) in their chromatographic study of the flavonoid extracts from spikelets of 23 strains of 14 Agropyron species. A definite parallel was found between morphological and chromatographic observations. They found that certain compounds were either missing in an entire section of the genus or marked a particular section by their presence.

In a study of karyotypes Schulz-Schaeffer and Jurasits (1962) found that no satellite chromosomes were observed in the species of the section Agropyron which they studied. Certain indicator chromosome types were found in species of the other sections except in A. triticeum of the section Eremopyrum. In a general way this is in accord with the findings of this serological investigation in that only the section Agropyron is distinct and the sections Goulardia and Holopyron are very similar.

The investigations of others mentioned above partially substantiate the validity of some sections and the similarity of the

sections Goulardia and Holopyron. This agrees with the findings of this study. For example, when A. donianum (#32-1) and A. brachyphyllum (#3-2), section Goulardia, were compared with A. elongatum (#37-5) and A. trichophorum (104-3), section Holopyron, they were serologically identical as determined by the absorption test. A comparison of A. hirsutum (#A1-S), section Eremopyrum, and A. spicatum (#75-3), section Holopyron, showed a difference index of 0.02. Neither of these two species is more closely related to other species in its respective section. The difference index resulting from a Four-way test of A. semicostatum (#64-8), section Goulardia, and A repens (#146-S), section Holopyron, is 0.02. Other observations casting doubt on the validity of some sections could be pointed out from data of the Four-way test, but in general it shows that many species are serologically more closely related to some species in another section than to some in their own section. The serological relationships revealed by this study disagree with the 4 section classification of Holmberg (1926). The section Agropyron appears to be a valid group, but sections Goulardia and Holopyron should be treated together. The section Eremopyrum was not investigated adequately to come to conclusions regarding its validity. In spite of the results revealed by this study it should be pointed out that serology is but one of many tools used in determining evolutionary relationships.

A serological investigation of grasses has not been success-

fully made prior to this study. This necessarily means that this investigation is a pilot study and establishes a foundation on which future research may be based. A major contribution of this study has been the Four-way test which is based on comparison with respect to four sources of antisera, one produced in response to injected extracts from a species of each of the four sections of the genus. With this work concluded it is clear that the four species used for antibody production for the Four-way test were not the best choice because they were too closely grouped as shown in the model (Figure 20). It would have been better to select species that occupy the extreme positions without regard to section classification but, however, the extreme species were not known prior to completion of this research. Because the 4 species used in the Four-way test to stimulate antibody production were rather closely grouped some of the relationships determined by the test may be exaggerated.

SUMMARY

Forty-five strains of 41 species of the genus Agropyron were tested for their serological relationships as determined by a specially designed test called here Four-way test. In this test antigens of each species were allowed to react with 4 different sources of antiserum. The reactions of each species were compared with those of each of the others treated in this study. From these data a difference index, which has been shown to represent adequately their serological relationship, was determined for each comparison. In several instances species which had differences index numbers of zero, indicating identical serological reactions, were somewhat different in morphology. The antiserum was produced to only 4 of the 41 species, and thus it may be that species which were serologically identical in these tests may give slightly different reactions when allowed to react with other sources of antiserum.

Those species comparison resulting in a difference index of zero or 0.02 in the Four-way test were tested in additional ways--absorption or adjacent plating. The absorption test revealed that 4 species, A. brachyphyllum, A. donianum, A. elongatum (hexaploid), and A. trichophorum were serologically identical in reaction to antiserum produced in response to A. sibiricum. Two strains of A. desertorum and hexaploid A. cristatum were also found to be serologically identical by these tests.

To adequately demonstrate the serological relationships of the species tested, a 3-dimensional model was constructed. The 3-dimensional representation shows a possible serological evolution of the genus to be

mainly in two directions. The crested wheatgrass complex occupies one of the extreme groups, and 4 species--the most remote of which is A. pycnanthum--the other.

The serological testing of 3 strains of A. elongatum with 3 different ploidy levels revealed that they were not as closely related to each other as they were to other species. It is proposed that these strains probably should be recognized as different species.

Seven taxa of section Agropyron were investigated by means of the antigen absorption test. Two strains of A. desertorum (#s 25-9 and 30-9) had identical serological reactions. The hexaploid strain of A. cristatum had serological reactions identical to those of the two strains of A. desertorum. By antigen absorption of diploid A. cristatiforme antiserum, these three strains resembled A. cristatiforme less than the other crested wheatgrasses tested. A. imbricatum and A. mongolicum resembled A. cristatiforme more closely than the other 4 taxa, but they were not serologically identical. A. sibiricum was intermediate in relationship to A. cristatiforme if compared with the others.

The crested wheatgrasses treated in this work were compared morphologically. Scale drawings were made of the glumes, lemmas, paleas, and anthers. These structures were smallest in the diploid A. cristatiforme, largest in the hexaploid A. cristatum and intermediate in the tetraploids. Some morphological differences were found in the two strains of A. desertorum, which had identical serological reactions in this treatment.

Evidence found in this study suggests that some sections of Agropyron, proposed by Holmberg (1926), are unnatural groupings. The species A. hirsutum (section Eremopyrum) resembled A. spicatum (section Holopyron) more closely serologically than any other species of the same section. A. spicatum was more closely related to A. hirsutum than to any other species tested except A. elongatiforme. The species A. brachyphyllum and A. donianum, both of the section Goulardia, A. elongatum (hexaploid strain) and A. trichophorum, of the section Holopyron, were found to be serologically identical when compared in this research. It appears that the sections Goulardia and Holopyron should be grouped together and that section Agropyron is a valid and distinct group. Section Eremopyrum was not investigated adequately to come to a conclusion regarding its treatment.

The proposal that A. sibiricum is of hybrid origin, resulting from a cross of A. repens and A. cristatiforme (Troitskii, 1932; Tronickij, 1959), was tested. The data are not conclusive but apparently A. sibiricum is not of the origin indicated. When reacted with A. sibiricum antiserum, all precipitate lines formed with both A. repens and A. cristatiforme were in common with some of those of A. sibiricum. A. sibiricum had antigen-antibody reactions which A. repens and A. cristatiforme did not have. If this species is of hybrid origin as proposed, it has a hybrid antigen; that is, one not occurring in either parent. This phenomenon has been demonstrated by several workers (McGibbon, 1944; Irwin, 1951; Alston and Turner, 1963).

Knowles (1955) and Dewey (1963), have considered that the different ploidy levels of A. cristatum were close enough to be referred to as the same species. Tests made in the present study do not support this premise. The diploid, referred to in this work as A. cristatiforme, appears to warrant recognition as a distinct species as Sarkar (1956) described it.

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