MONTANA STATE UNIVERSITY

Serology as a technique to detect major genetic differences in the genus Agropyron Gaertn
by John Edward Ericson

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:
Twenty-six collections of twenty Aqropyron species were examined serologically using antigenic material from leaves. Antisera were produced in New Zealand white rabbits. Double-diffusion serological reactions in agar were used to interpret hybrids, putative hybrids and relations among plants of Aqropyron which contain the so-called B genome. Set notation is developed as a tool for describing and interpreting antigen-antibody precipitate lines in double-diffusion gel plates. Based on serological evidence four of the five putative hybrids studied are apparently seifs rather than hybrids. One putative hybrid could not be distinguished as either a self or a hybrid by serological means.

Data are presented which illustrate the complexity of the interaction between two genetic systems. Serologically four kinds of differences were found; 1) rabbit to rabbit differences illustrated by rabbits 009, 010 and 10-3 which did not respond the same to antigenic material from plant 10-3; 2) tissue to tissue differences illustrated by the comparison of the X-Re of antigenic material from culms and spikes of plant 10-3 with the R-Re of leaf antigenic material from plant 10-3; 3) plant to plant differences within a species illustrated by the A. intermedium intraspecific putative hybrid, the A. cristatiforme (16-1) X-Re with antiserum from rabbit 10-3 and the A. dasystachyum X-Re with antiserum from rabbit 147-s, and 4) species to species differences similar to those reported by Creel (1964).

A. spicatum (n=14) is considered a variable autotetraploid with genome formulae of SSSS to SSSxSx , where x is a varying amount of change in the different populations A. spicatum. A. arizonicum is considered to be a segmental allotetraploid and is given the genome formula of SSSBSB based upon serological data and supported by cytogenetic information.

Cryptic structural hybridity may not be distinguished in hybrid analysis or karyotype analysis. Morphological information has not shown the relationships of the Aqropyron. Serological information may be an important source of information in a biosystematic program but should not be considered as either superior or inferior to other systematic data.

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by

JOHN EDWARD ERICSON

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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in

Genetics

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This thesis is dedicated to my wife, Jan, without whose encouragement and understanding this study could not have been completed.

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## ABSTRACT

Twenty-six collections of twenty Agropyron species were examined serologically using antigenic material from leaves. Antisera were produced in New Zealand white rabbits. Double-diffusion serological reactions in agar were used to interpret hybrids, putative hybrids and relations among plants of Agropyron which contain the so-called B genome. Set notation is developed as a tool for describing and interpreting antigen-antibody precipitate lines in double-diffusion gel plates. Based on serological evidence four of the five putative hybrids studied are apparently selfs rather than hybrids. One putative hybrid could not be distinguished as either a self or a hybrid by serological means.

Data are presented which illustrate the complexity of the interaction between two genetic systems. Serologically four kinds of differences were found; 1) rabbit to rabbit differences illustrated by rabbits 009, 010 and 10-3 which did not respond the same to antigenic material from plant 10-3; 2) tissue to tissue differences illustrated by the comparison of the X-Re of antigenic material from culms and spikes of plant 10-3 with the R-Re of leaf antigenic material from plant 10-3; 3) plant to plant differences within a species illustrated by the A. intermedium intraspecific putative hybrid, the A. cristatiforme (16-1) X-Re with antiserum from rabbit 10-3 and the A. dasystachyum X-Re with antiserum from rabbit 147-s, and 4) species to species differences similar to those reported by Creel (1964).

A. spicatum (n=14) is considered a variable autotetraploid with genome formulae of SSSS to $SSS_xS_x$, where x is a varying amount of change in the different populations of A. spicatum. A. arizonicum is considered to be a segmental allotetraploid and is given the genome formula of $SSS_BS_B$ based upon serological data and supported by cytogenetic information.

Cryptic structural hybridity may not be distinguished in hybrid analysis or karyotype analysis. Morphological information has not shown the relationships of the Agropyron. Serological information may be an important source of information in a biosystematic program but should not be considered as either superior or inferior to other systematic data.

## INTRODUCTION AND LITERATURE REVIEW

In 1960 a research program concerned with the biosystematics of the genus Agropyron was initiated at Montana State University under the direction of Professor Jurgen R. Schaeffer. The present study was undertaken to investigate serology as a taxonomic technique and to detect genetic differences in Agropyron by means of serology.

The genus Agropyron contains approximately 150 species, of which 100 are native to Europe and Asia, 30 are native to North America and the remainder are found in South America, Australia, New Zealand and Africa.

Many species of Agropyron are of considerable economic importance. Many are excellent forage species and others are serious pests. Agropyron has been regarded as an artificial genus by numerous workers (Nevski 1934, Parodi - cited in Connor 1954, and Stebbins and Walters 1949). Stebbins and Walters were of the opinion that the established species names should be retained until adequate cytogenetic and morphological data permit the development of a more natural system. Beetle (1961) suggested that detailed field studies in the regions where the species are native are necessary before the true answers to the classification puzzles can be obtained. It is generally agreed that the status of the genus Agropyron is confused and that many of the species are not adequately defined within the taxa.

Parodi (cited by Connor, 1964) considered the genus Agropyron partly artificial in that separation of the South American Agropyron and Elymus could not be achieved by morphological characteristics. Gould (1947) placed the California species of Agropyron, Sitanion, Hystrix and Elymus

into one genus. Stebbins and Walters (1949) concluded that there was not enough evidence to determine the boundary of the genus.

Information of a biochemical nature has proven useful in taxonomic investigations (Alston and Turner 1963). Modern taxonomy takes into account all forms of information, especially those which are capable of detecting genetic differences. Serological techniques measuring genetic variation by antigen-antibody reactions may offer insight into problem areas of plant systematics.

The precipitin reaction was discovered by Kraus in 1897 (cited by Fairbrothers and Johnson 1964). Nuttall (1901) was the first to show the possible uses for this method in taxonomic investigations. Much serological work has been done with zoological classification. The earliest work at Montana State University was done by Martin and Cotner (1934) who investigated the serological properties of moth thorax antigens. They suggested that "...the application of immunological reactions in the determination of relationships between species of various organisms has brought to the hand of the systematist a new and exact method of determining phylogenetic relationships...supplementing...other studies in the classification of various biological groups".

The use of serology in plant investigations has been less advanced than in animal investigations. This may be explained by historical events in serobotany and the fact that serology began as a zoological method. Furthermore animal antigens, unlike plant antigens, are not accompanied by compounds such as alkaloids, which may be harmful to the experimental animal.

Kowarski (1901, cited in Hall 1959) was perhaps the first to use plant materials as antigens when he caused the production of antibodies in rabbits using heat resistant albuminose from wheat. These antibodies reacted strongly with wheat albumin extracts but weakly or not at all with extracts of rye, barley, oat and pea. He found what could be considered an antisystematic reaction when the reaction with the pea extract was stronger than that with the oat extract. Ballner and Burrow (1910) found rye serologically more closely related to wheat than to barley. This relationship is in agreement with Godley's view of the Triticeae (Hordeae) comparium (Godley 1951).

Rives (1923 a,b) and Green (1926) found the ability to graft successfully was accompanied by a distinct serological similarity in species of each of the taxa Vitus, Citrus, Rosaceae and Solanaceae.

Phytoserological activity reached a peak with the publication of Der Königsberger Serodiagnostische Stammbaum by Mez and Ziegenspeck (1926). This phylogenetic structure based on serology was severely criticized by Gilg and Schurhoff (1927) of Berlin. Moritz (1964) states "The...feud between the Königsberg school of Mez and the Berlin school of Gilg and Schurhoff...did much damage to the prestige and activity of the method among botanists". A thorough review of this conflict is given by Chester (1937). A few researchers, Johnson at Rutgers and Moritz at Institut für Pharmakognosie, Kiel, Germany, continued to experiment with the methods involved with plant serology.

Recent serological work deals mostly with small groups of closely related taxa at the family level or lower. Gell, Hawkes and Wright

(1960), working with the tuberous members of the genus Solanum, were able to show relationships corresponding to relationships based upon morphological and cytogenetic data. Lester (1964) expanded and continued this work. Johnson (1954) and Johnson and Fairbrothers (1965) found Liriodendron to be serologically distinct from the rest of the Magnoliaceae. Fairbrothers and Johnson (1964) and Fairbrothers (1966) studied the Cornaceae complex serologically and were of the opinion that the separation of the complex into Cornaceae with one genus and Nyssaceae with two genera was justified.

Brown and Lester (1965) found what they consider to be a probable error in the taxonomic placement of one species of green algae based on serological reactions.

Hall (1959) studied a stabilized polyploid hybrid between wheat and rye (Triticale) and found that all proteins identified in the wheat could be traced to the hybrid. Specific Triticale antigens were not found.

Sawyer (1967) was not able to separate the Montana species of Delphinium serologically. Ericson et al. (1967) found Populus tremuloides serologically distinct from other Populus studied.

Lester et al. (1965) found serological data conservative in that it delineated the genus but did not help to distinguish between the fourteen species of Baptista studied. It is their opinion that "there is no clear justification for regarding serological data as intrinsically either superior or inferior to other systematic criteria".

The discovery of the double-diffusion precipitin reaction in gels (Ouchterlony 1948) made a significant contribution to serotaxonomic

studies.

Ouchterlony (1964) discusses four types of serological reactions found in double gel-diffusion plates. The type I or identity reaction occurs when precipitate lines join in a smooth curve and are interpreted as being formed by identical or nearly identical antigens reacting with the same antibodies. The type II or nonidentity reaction occurs when lines cross another line and are interpreted as being caused by different reacting antigens. Type III or partial identity reactions cause the formation of a spur rather than a smooth curve and are taken to mean that nonidentical but similar antigens are reacting with the same or a closely related antibody. In the type IV or reaction-inhibition reactions, precipitate lines do not join in a meaningful pattern. The number of bands may not indicate the maximum number of antigen-antibody systems present since some of the bands may be obscured by others (Fairbrothers 1966, Kwapinski 1965). The antigenic material used to produce an antiserum is referred to as the "reference antigen" (R-Ag) and all other antigenic material which will react with this antiserum is considered a "crossreacting antigen" (X-Ag) (Williams 1964). The reaction observed when the antiserum is reacted with the reference antigen is the "reference reaction" (R-Re) while the reaction observed when the antiserum is reacted with any other antigenic material is considered a "cross reaction" (X-Re) (Fairbrothers 1966). Theoretically no cross reaction can produce more lines than the reference reaction. It may produce as many lines but probably will produce fewer (Moritz 1964).

The objectives of this study were: 1) to determine the serological behavior of known hybrids and to use the results to determine the success or failure of hybridization attempts and to make inferences about the relationships of the plants involved; and 2) to determine the serological relationship of the B genome in some of the tetraploid species of *Agropyron* such as *A. arizonicum* and *A. caninum* in relationship to the S genome found in the diploid *A. spicatum*.

## MATERIALS AND METHODS

The concept of serology is based upon the fact that the introduction of a foreign substance of biological origin (antigen) into the circulatory system of an animal may give rise to the production of substances (antibodies) in the blood which are capable of affecting the introduced antigen in a number of ways. This production of antibodies is the response of an organism to the gene products of another, nonrelated organism. In this study, the antibody producers were rabbits and the antigen sources were plants.

Antigen preparations were made from 26 collections of 20 species of Agropyron (Table I) grown in the Montana State University experimental grass nursery at Bozeman, Montana or in the Plant and Soil Science greenhouse. Two native strains not grown in the nursery are included in Table 1. This nursery was established in the spring of 1961 and samples were taken the springs of 1963, 1964, 1965 and 1966. To prepare the antigens, young leaves were taken from the culms of mature plants with new vegetative growth or from seedling plants which had reached a height of 15 mm. When seedlings were used, several plants were taken as a composite lot in order to have sufficient extract. In the latter case any genetic or environmentally induced differences between plants of a collection would be confounded because of the mixing of antigens from more than one plant. The leaf material was identified as to source and taken to the laboratory where it was washed twice in distilled water then blotted dry. After blotting, the leaves were placed in a metal cylinder one inch in diameter and four inches long, chopped firmly by hand and then pressed

Table I. Sources of 26 strains of 20 Agropyron species, growing in the Montana State University grass nursery, used in these studies.

| Species | MSU Field No. | Source |
|---|---|---|
| A. arizonicum Scribn. et Smith | 2-8<br>2-S* | 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 (Schulz-Schaeffer and Jura 1967) MONT 59,277. |
| A. brachyphyllum Boiss, et Haussk | 3-1<br>3-4 | Collected by H. S. Gentry, east base of Kuhe Zard, Charmahal, Iran, November 9, 1955. 2n=42 (Schulz-Schaeffer and Jurasits, 1962) MONT 59,278. |
| A. caninum (L.) Beauv. | 6-3<br>6-5 | Seed from Botanic Gardens, Budapest, Hungary via Gaterslaben, Germany. Received through Institut Für Pflanzenbau und Pflanzenzüchtung, Göttingen, Germany in January, 1960 as field Nos. 4-1957 and 131-1958. 2n=28 (Allderdice 1965) MONT 59,279. |
| A. ciliatiflorum Roshev. | 9-8 | Collected by H. S. Gentry at Paghman, Kabul Province, Afghanistan, September to October 1953. P.I. 207,452. 2n=28. (Schulz-Schaeffer and Jurasits, 1962). MONT 59,280 |
| A. cristatiforme Sarkar | 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. MONT 59,281. |
| | 16-1 | 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. n=7 P.I. 19,536. Variety "Fairway". MONT 59,285. |

Table I (cont'd)

| Species | MSU Field No. | Source |
|---|---|---|
| A. dasystachyum (Hook.) Scribn. | 147-S | P.I. 236,663. Received through Dr. Douglas Dewey, ARS, USDA, Logan, Utah 2n=28. |
| | A.d. | Collected by D.D. Collins, Bear Paw Mountains near Havre, Montana, in the Montana State University grazing allotment on August 8, 1962. n=14 (Collins 1965) MONT 58,690. |
| A. desertorum (Fisch.) Schult. | 30-10 | Alma-Ata, Russia, 2n=28 (Creel, Ericson, and Schulz-Schaeffer 1964). |
| A. elongatum (Host.) Beauv. | 36-5 | Introduced by N.I. Vavilov from the Saratove Institute, Russia, on April 8, 1932. P.I. 98,526. p-2326. Strains "Nebraska 98,526" and "Blue Type". n = 35,36. MONT 59,298. |
| | 36-1 | 2n = 42 (Schulz-Schaeffer and Jura 1967). |
| A. intermedium (Host.) Beauv. | 34a-3 | Received from Botanic Gardens, Scientific Academy of USSR, Taschkent, Karamurtskaja, Russia, as A. elongatum, through Dr. Christian Lehmann, Institut für Kulturpflanzenforschung, Gatersleben, Germany, on August 1, 1959. Morphological characteristics of spikes and leaves, early flowering period and rhizomatous characteristics indicate that it is rather A. intermedium. 2n = 42. B-67. MONT 59,300. |
| | 35a-7 | Volunteer in nursery (replacing A. elongatum 2n=14) 2n=42 MONT 59,297. |
| | 44-2 | 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, |

Table I (cont'd)

| Species | MSU Field No. | Source |
|---|---|---|
| | | Germany, on May 27, 1960. MONT 59,305. |
| A. latiglume (Scribn. et Smith) Rydb. | 48-2 | Collected by Drs. F.J. Hermann and B.M. Leese at Middle Fork of Sheeps Creek on road to Spirit Lake (Ashley National Forest), Daggett Co., Utah, at 9,000 ft. elevation in lodgepole pine meadow during July to September 1955. P.I. 232,117. MONT 59,310. |
| A. junceum (L.) Beauv. | 46-5 | Received from Botanic Gardens Frankfurt-Main, Germany, on March 28, 1960. B-72. n = 34, 34 + IU. MONT 59,307. |
| A. panormitanum Parl. | 50a-2 | Received from Colonial Botanic Gardens, Palermo, Italy, July 1951. P.I. 197,569. 2n=28. (Schulz-Schaeffer and Jurasits 1962). n = 14. MONT 58,421. |
| A. pycnanthum G.G. | 19-1 (1965) | Sent by Mrs. Yvonne Cauderon from Clermont-Ferrant, France. Origin: Riec sur Belon, Department Finisterre, France. |
| A. riparium Scribn. et Smith | 62-2 | Received from seed collection of the Institut für Pflanzenbau und Pflanzenzüchtung, Göttingen, Germany, in January 1960 as Field Nos. 60-1957 and 171-1958. MONT 59,317. |
| | 62a-4 | Collected by Dr. R.G. Johnson as A. smithii Rydb. near Canyon City, Grant County, Oregon, in area of 12 inch annual rainfall at elevation of 3,000 ft. in 1933. P-2415. Variety "Sodar." 2n = 42. (Schulz-Schaeffer and Jurasits 1962). MONT 59,318. |
| A. semicostatum (Steud.) Nees | 65-4 | Received from seed collection of Institut für Pflanzenbau und Pflanzenzüchtung, Göttingen, Germany, |

Table I (cont'd)

| Species | MSU Field No. | Source |
|---|---|---|
| | | January 1960, as Field Nos. 59-1957 and 170-1958. n = 14. MONT 59,321. |
| A. sibiricum (Willd.) P.B. | 68-5 | 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. MONT 59,322. |
| A. spicatum (Pursh.) Schribn. et Smith. n=14 | 79-2 | Grass and legume Standard Accession list. U.S.D.A. Soil Conservation Service. Pullman, Washington, February 1955. P-7845, n=14 (Ericson) MONT 59,328. |
| | n=7-S* | Collected at the Belgrade transmission tower, Belgrade, Gallatin Co., Montana on July 28, 1962. Large population over gravely bench. n=7 (Collins 1965) MONT 58,686. |
| A. subsecundum (Link) Hitch. | 185 | Collected by D.D. Collins at Flathead Pass, Bridger Mountains near Bozeman, Gallatin Co., Montana on September 10, 1962. Widespread on north slopes at 7,200 ft. elevation. n=14 (Collins 1965) MONT 58,687. |
| A. trachycaulum (Link) Malte. | 93-2 | Collected by the U.S.D.A. Forest Service near Beebe, Montana 1933. Variety "Primar" P-2535, n=14 (Allderdice 1965) MONT 59,338. |
| A. triticeum Gaertn. | 125-S* | Collected by Dr. W.E. Booth near Gardner, Montana in 1952. n=7 (Schaeffer 1964) |

Explanation of Accession Numbers:

MONT = Herbarium of Montana State University, Bozeman, Montana. (Lanjouro and Stafleu 1954).

Table I (cont'd)

Explanation of Accession Numbers (cont'd)

P & A = Seed collection of the U.S.D.A., Soil Conservation Service, Pacific Region, Plant Material Center, Washington State University, Pullman, (P), Washington, and SCS Southwestern Region, Albuquerque (A), New Mexico.

P.I. = Seed collection of the U.S.D.A., A.R.S., New Crops Research Branch, Crops Research Division, Plant Introduction Stations, Western Region, Pullman, Washington and North Central Region, Ames, Iowa.

U = Univalent.

n, 2n = As is customary in reporting chromosome numbers, n-numbers represent data from meiotic and 2n-numbers data from mitotic studies.

s* = Indicates material from several seedlings which was bulked to have sufficient material for use.

6-3 = Plant number three in accession number 6.

with 2,500 psi using an hydrolic jack. Approximately 50 grams of this material could be squeezed at a time, although smaller amounts were treated satisfactorily. Fifty grams of this material yielded up to 10 ml. of crude extract.

The crude extract was placed in small sterile vials in 1 ml. aliquots and was immediately frozen at -20°C. until used. No preservative was used. To prepare the crude extract for injection, the material was thawed and centrifuged two minutes at 1,500 rpm (325 G) to remove chloroplasts and fragments of cell wall. All extracts were discarded after having been thawed the second time due to deterioration of antigenic material.

Antisera were produced in New Zealand white rabbits in response to injected extracts. The injections were given subcutaneously near the lymph glands in the shoulders and in the lumbar region. Each injection aliquot was divided into four portions with one portion injected into each of the four regions of the rabbit. No adjuvant was used. The injections were given in weekly series beginning with 0.5 ml., then 1 ml., 2 ml., 3 ml. and a final 4 ml. injection. Booster injections of 1 ml. were given approximately once a week to maintain a high antibody level. Rabbits were kept in individual cages. Rabbits of both sexes were used.

Seven days following the last injection, and weekly after each booster as needed for study, the rabbits were bled from the lateral ear vein. The arteries and veins were dilated with xylene or acetone. The lateral ear vein was opened with a sterile razor blade and the blood was collected in a sterile petri dish. Forty to 60 ml. of whole blood were

taken at each bleeding. Direct pressure with sterile cotton was sufficient to allow the wound to close. Care was taken to remove all traces of xylene from the ear.

The blood was allowed to coagulate in the covered petri dish for about three hours and then the serum was centrifuged at 3,000 rpm (1500 G) for twenty minutes to remove remaining erythrocytes, leucocytes and platelets. The serum was divided into 1 ml. portions, placed in small vials and frozen at -20°C. No preservative was added. Each container was marked with the date of bleeding and the rabbit number. Each bleeding was treated as a separate lot. After being thawed twice, the serum was either used or discarded.

A modification of the gel-diffusion technique described by Ouchterlony (1948) was used to detect differences between plants. The gel-diffusion plates were 90 mm. in diameter with agar poured to a depth of approximately 1.7 mm. The agar was prepared as a 0.6% (W/v) solution of "Ionagar" No. 2 (Oxoid Consolidated Laboratories, Inc., Chicago Heights, Ill.) dissolved in a 0.9% saline solution buffered to a pH of 7.2 with sodium barbitol (vernol) and HCl in distilled water. One part in 5,000 of sodium azide was added as preservative. After dissolving the agar in the buffered saline, 11 ml. of the solution were pipetted onto sterile petri dishes. The prepared plates were allowed to stand for several hours before the wells and trench were made. The plates were cut following the design of Creel et al. (1965) and filled with the appropriate serum and antigen extracts. To test several antisera against a single antigen complex a plate with eleven wells was used (Figure 15). The wells were

made with a #3 cork borer tapered to the inside and were 8 mm. in diameter with the outside wells being 8 mm. from the center wells. The three center wells were filled with antigenic material and the outside wells filled with antisera. The plates were allowed to stand 48 hours at room temperature (20°C. to 25°C.). During this incubation period the antigens and antibodies diffused through the gell. Precipitate lines formed where specific antigens and antibodies met in appropriate concentration.

At the end of the incubation period, the gel-diffusion plates were read immediately or stored at 4°C. up to a week until they could be interpreted. The gel-diffusion plates were read in a darkened room by allowing fluorescent light to pass through the agar at an oblique angle, making the lines clearly visible.

After the initial reading, the plates were flooded with distilled water for six or more hours to wash out unreacted proteins such as gamma globulins and hemoglobins. The distilled water was removed by vacuum and immediately replaced with 0.9 (w/v) NaCl in distilled water. The saline solution was changed two or three times and allowed to stand overnight to insure good flushing of residual globulins (Jutila 1966). At each change of fluid, a crop of merthiolate was added to each plate to retard bacterial and fungal growth. The agar was floated out of the petri dish with distilled water and onto a lantern slide (3 1/4 x 4 1/4) after which the agar was allowed to dry. At this point the slides could be stored indefinitely.

To prepare the slides for staining, they were pretreated by

flooding with 2% glacial acetic acid (2/98 v/v) and held for five minutes. The slides were then stained in 0.5% amido schwartz solution [0.5% amido schwartz(National Aniline) dissolved in 100 ml of 9 parts methanol to 1 part glacial acetic acid (9/1 v/v) then filtered before use] for one hour. The slides were next destained by washing in 9/1 (v/v) methanol / glacial acetic acid solution four to five times. The slides were left in the last wash overnight and then dried (Jutila 1966). The stained slides were projected onto a screen and compared with the original interpretation. All plates were run in duplicate. The four expected precipitate patterns discussed by Ouchterlony (1964) are diagramed in Figure 1.

In the present study four hybrids discussed by Allderdice (1965) and five putative hybrids were studied (Table II) by comparing leaf antigenic material from the parent plants with leaf antigenic material from the hybrid or putative hybrid. The parent plants of hybrid A. brachyphyllum X A. riparium were not available and plants of the same species were used in the comparison. All hybrids and putative hybrids were tested with antisera from rabbits 10-3 and 36-5. The species and animals used for antibody production are listed in Table III.

To test the serological relationship of some species of Agropyron containing or suspected of containing the "S" genome, four antisera were absorbed or partially absorbed with leaf antigenic material from a local population of A. spicatum n=7 from Belgrade, Montana. Antisera were absorbed by mixing antigenic material with the antisera and allowing the mixture to incubate at 37°C. for four hours. The antigenic materials re-

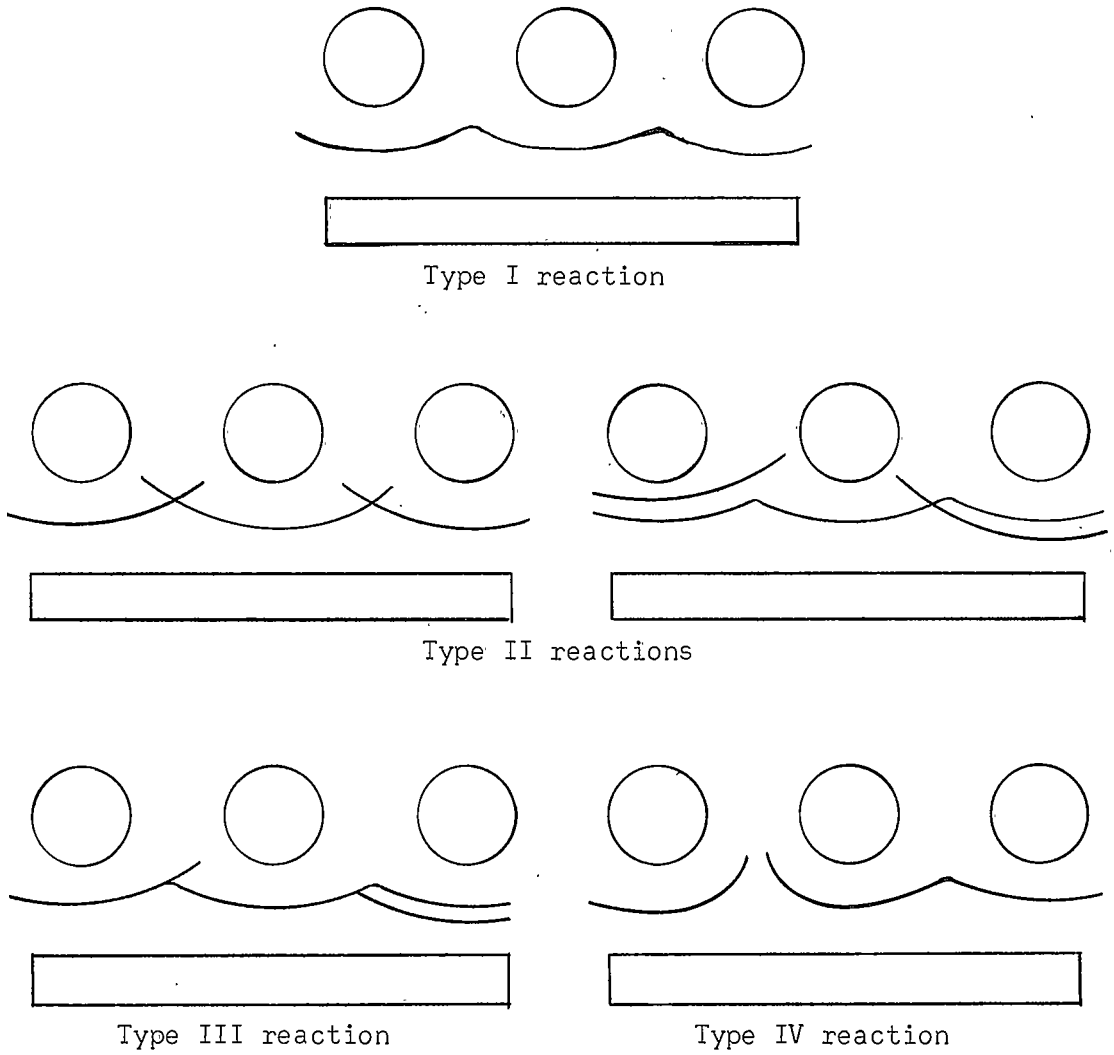



Fig. 1 Drawing of Ouchterlony's type I, type II, type III and type IV reactions. No known type IV reactions were observed.

Table II. Four known hybrids and five artificial putative interspecific and intraspecific *Agropyron* hybrid plants which were compared serologically with their parent plants.

| Female Parent | | Male Parent | |
|---|---|---|---|
| Species | Accession No. | Species | Accession No. |
| *A. brachyphyllum** | 3-1 | *A. riparium* | 62-2 |
| *A. caninum** | 6-3 | *A. latiglume* | 48-1 |
| *A. caninum** | 6-5 | *A. riparium* | 62a-4 |
| *A. ciliatiflorum* | 9-8 | *A. cristatiforme* | 16-1 |
| *A. intermedium* | 34a-1 | *A. intermedium* | 44-2 |
| *A. intermedium* | 34a-1 | *A. junceum* (2n=70) | 46-1 |
| *A. intermedium* | 35a(1963)-7 | *A. elongatum* | 36-1 |
| *A. semicostatum** | 65-4 | *A. trachycaulum* | 93-2 |
| *A. spicatum* (2n=28) | 79-2 | *A. pycnanthum* | 19(1965)-3 |

* Hybrids discussed by Allderdice (1965).

Table III. *Agropyron* species used to induce antibody production in New Zealand rabbits.

| Species | M.S.U. Field No. | Rabbit No. |
|---|---|---|
| *A. cristatiforme* | 10-3 | 10-3* |
| | 10-3 | 009 |
| | 10-3 | 010 |
| *A. sibiricum* | 68-5 | 68-5* |
| | 68-5 | 011 |
| *A. desertorum* | 30-10 | 012 |
| | 30-10 | 025 |
| | 30-10 | 026 |
| | 30-10 | 027 |
| | 30-10 | 028 |
| *A. panornitanum* | 50a-7 | 50a-7* |
| *A. triticeum* | 125-S | 001* |
| *A. dasystachyum* | 147-S | 147-S* |
| *A. elongatum* | 36-5 | 36-5 |

* Reported in Creel 1964.

acted with their specific antibodies forming a precipitate. After incubation the precipitate was spun down by centrifugation (325 G.) and discarded. The absorbed antiserum was used to test for antigenic material not contained in A. spicatum n=7.

To estimate the amount of homozygosity and heterozygosity present in the plants studied, an estimate of the breeding behavior was necessary. One method used to study breeding behavior is to determine the amounts of seed set in open-pollination versus that set under conditions of forced self-pollination. To determine the amount of self-fertility of various Agropyron species, tests were carried out during the summers of 1962, 1963, 1964, 1965 and 1966. Plants were isolated by means of isolation chambers (Creel and Schulz-Schaeffer 1963), individual heads were placed in glassine bags, or groups of five or more spikes were placed in heavy waterproof craft paper bags designed for corn pollen. In the latter case, the corn pollen bags were supported by heavy bamboo stakes, with a wire insert to keep the bag open and allow free movement of pollen. The bags were agitated daily to insure adequate pollen distribution. Self-pollination and open-pollination seed set was counted for each plant except for the year 1962 when only open-pollination counts were made and the year 1965 when only self-pollination counts were made. The percentage seed set, percentage open-pollination, percentage self-pollination and the percentage self-fertility were calculated for each strain.

Set notation can be used to describe serological reactions. The definitions for symbols and operations useful and applicable to a serological system are established below:

$V$ denotes the universal set. In this study V has meaning only when qualified by a subscript.

$V_{10-3}$ denotes the finite set of all possible antigen-antibody reactions with antiserum 10-3. The subscript 10-3 refers to the antisera used.

$\emptyset$ denotes the empty set.

$A$ denotes the set of antigens from a particular plant which will cross-react with an antiserum. Other capital letters are similarly defined. $A = \{X_1, X_2, \ldots, X_n\}$

$A'$ denotes the complement of A; it is the set of antigen-antibody reactions not found in the set A. This has meaning only in reference to a particular V.

$\cup$ denotes the union operation. Thus $A \cup A' = V_x$. The union of two sets is the set of all elements that are in at least one of them.

$\cap$ denotes the intersection operation. Thus $A \cap V = A$. The intersection of two sets is the set of all elements common to both sets.

$a_n$ denotes a subset of A. $a_n$ may contain 0, 1, 2, or more elements (antigenic substances). $a_n = \emptyset$ and $a_n = A$ are allowable.

$\supset$ "is a subset of the set". Thus $a \subset A$ and $A \supset a$ would read "a is a subset of the set A". Also $a \not\subset A$ would read "a is not a subset of the set A".

Precipitate lines are formed on a plate due to antigen-antibody interactions. When using set notation to describe these antigen-antibody interactions all connecting lines on a plate will be designated as a subset. For a comprehensive discussion of set notation see Fine (1965).

Moritz (1960) with antiphylaxis techniques characterized the reactions of a wheat-rye hybrid in the following form:

$$WR = wr_1 + wr_2 + wr_3$$

$$W = wr_1 + wr_2$$

$$R = wr_2 + wr_3$$

Set notation may be used to describe these results. Let WR be the set of antigenic material in the wheat-rye hybrid, W the set of antigenic material found in the wheat and R the set of antigenic material found in the rye. $wr_1$ and $wr_2$ are subsets of W, $wr_2$ and $wr_3$ are subsets of R and $wr_1$, $wr_2$ and $wr_3$ are subsets of WR. Each subset contains one or more antigens. The formula may then be written:

$$W \cup R = WR \quad \text{or} \quad \{wr_1, wr_2\} \cup \{wr_2, wr_3\} = \{wr_1, wr_2, wr_3\}$$

The expected pattern would be for the hybrid to contain all the antigenic material found in the female parent plus all the antigenic material found in the male parent. When antigens have been tested with more than one antiserum and within each antigen-antibody reaction a subset such as $a_1$ is described, there is no reason to expect $a_1$ found in $V_x$ to to be equal to $a_1$ found in $V_y$. Hybrid plants are expected to contain all the antigentic responses of both parents. This may be shown as the union operation. For example in the wheat X rye hybrid (Moritz 1960) $WR = W \cup R$.

A putative hybrid that is in fact the result of selfing should be identical to the seed parent in its serological response.

It is assumed that:

1. Cross reacting antigens are identical or nearly identical and indicate a phylogenetic relationship between these antigens.
2. No cross reaction can produce more lines than the reference reaction.
3. Antigens are not masked by dominance or epistatic effects.

An effort was made to duplicate the antiserum produced by rabbit 10-3 (Creel et al. 1965) with two unrelated rabbits (009 and 010) the same age. The rabbits were not from the same source as rabbit 10-3. The two rabbits were treated identically with antigenic material from plant 10-3. Identical results were not obtained. Four rabbits (025, 026, 027 and 028) were obtained from the same litter and treated identically with antigenic material from plant 30-10, A. desertorum. No differences in titer or number of lines could be detected.

## EXPERIMENTAL RESULTS

### I. Hybrids and Putative Hybrids

To test five controlled putative hybrids and four known hybrids (Allderdice 1965) two antisera were chosen as common testors. The use of two antisera as common testors was to obtain results which could be compared with each other. Each antiserum has antibodies which may or may not be found in any other antiserum. If antiserum specific for a hybrid or putative hybrid was induced, then only experiments using that particular antiserum could be compared. The antisera were chosen because both were capable of strong cross-reactions with all *Agropyron* antigenic material tested.

Four known hybrids analyzed by Allderdice (1965) were tested by plating the leaf extract of the parent plants next to the leaf extract of the hybrid. Three hybrids: *A. caninum* X *A. latiglume*, *A. caninum* X *A. riparium* and *A. semicostatum* X *A. trachycaulum* behaved as expected. When tested with antisera from rabbits 10-3 and 36-5 all lines were held in common with all three plants. (Figure 2).

The hybrid *A. brachyphyllum* X *A. riparium* was tested against a plant of the same collection of *A. brachyphyllum* (3-4) and a plant from another strain of *A. riparium* (62a-4). Type II reactions were found when tested against antiserum 10-3 and type III reactions were found when tested against antiserum 36-5. Possible intraspecific variation among plants of *A. brachyphyllum* and among plants of *A. riparium* is suggested by these data. This implies that whenever

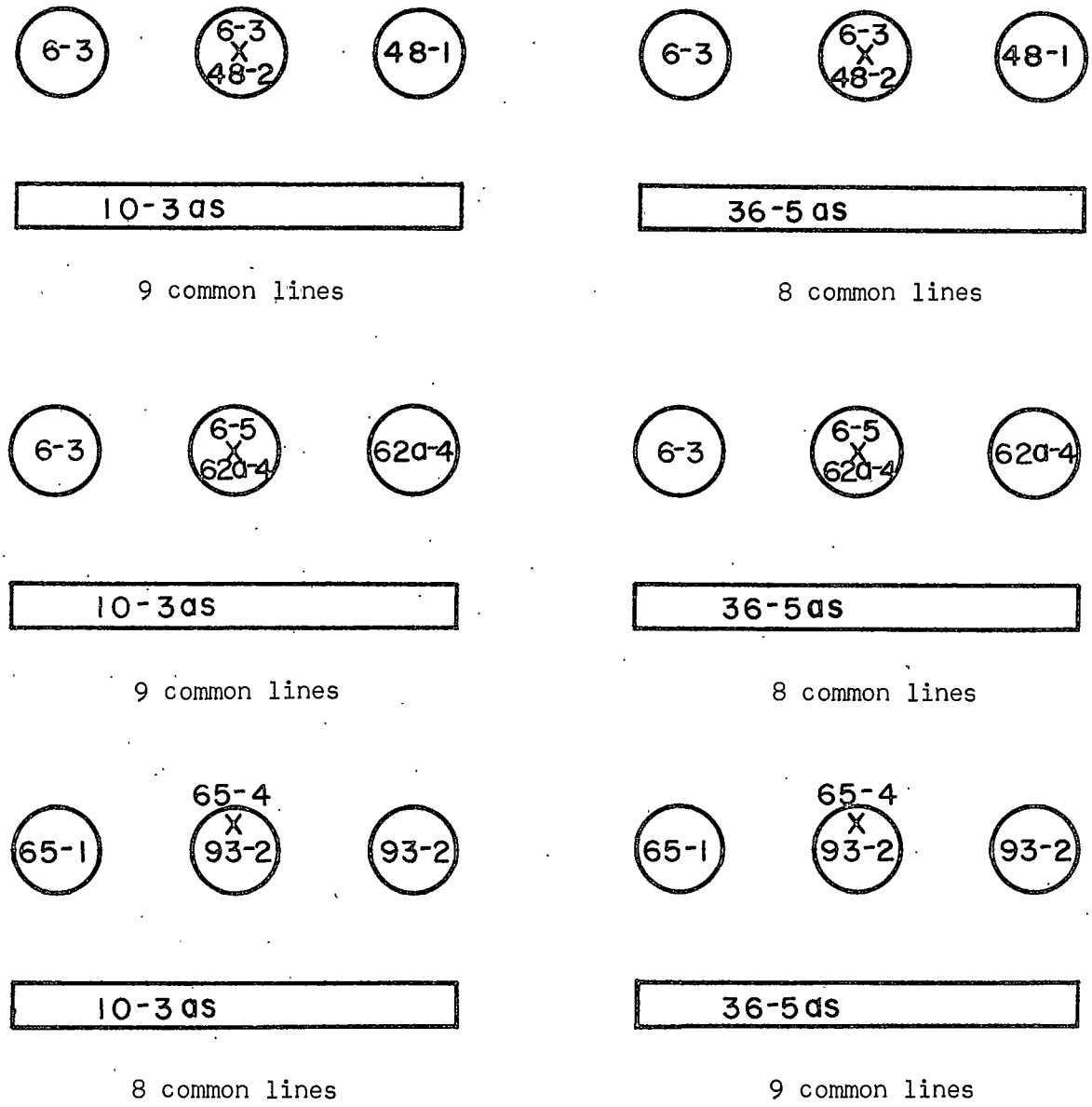



Fig. 2 Drawings of serological plates showing the design for the hybrids A. caninum X A. latiglume, A. caninum X A. riparium and A. semicostatum X A. trachycaulum. No differences were detected between parents and their hybrid.

possible the exact parents of a hybrid should be used. (Figure 3).

Five putative hybrids were tested against their known seed parent and the supposed pollen parent using antisera from rabbits 10-3 and 36-5.

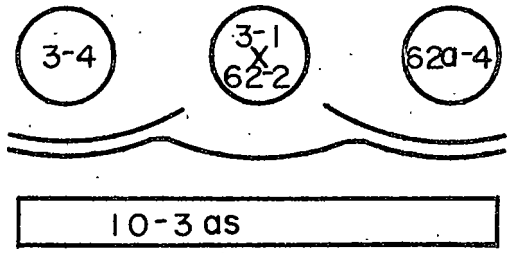
The putative hybrid A. intermedium (34a-1) X A. intermedium (44-2) and the seed parent (34a-1) showed one precipitate line that (44-2) did not have when tested against antiserum 10-3 and two precipitate lines not found in (44-2) when tested against antiserum 36-5 (Figure 4).

For the A. intermedium (34a-1) X A. intermedium (44-2) putative hybrid the antigen sets are assigned A. intermedium (34a-1) $I_1$, A. intermedium (44-2) $I_2$ and the putative hybrid $I_h$. Two universal sets $V_{10-3}$ and $V_{36-5}$ are involved. In the universe $V_{10-3}$ and the universe $V_{36-5}$, $I_1 \supset i_1$, $i_2$ and $I_2 \supset i_1$ where $i_1$ is the set of all common lines. If $I_h$ is a hybrid then it should equal $I_1 \cup I_2$. If $I_h$ is a self rather than a hybrid then it should equal the seed parent $I_1$. In both the universe $V_{10-3}$ and the universe $V_{36-5}$:

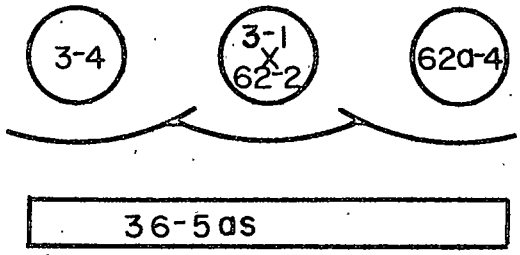
$$I_h = I_1 \cup I_2 = I_1.$$

Therefore $I_h$ is either a hybrid or it is a self. The hybridity of this plant cannot be determined by these tests.

The two collections of A. intermedium did not behave in an expected manner in that they were serologically different when tested with each of the antisera used as common testors. No differences between the parent plants were expected. Plant 34a-1 contained one

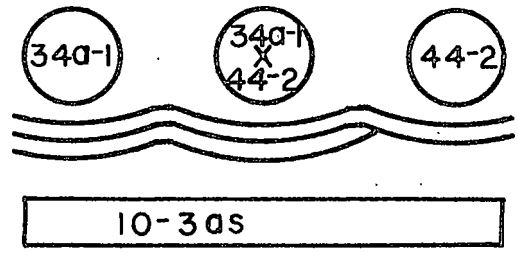



10 common lines

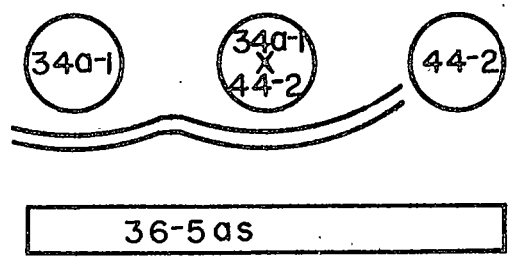



9 common lines

Fig. 3 Drawing of serological plates showing the differences observed in the hybrid A. brachyphyllum X A. riparium. Note the type I, II and III reactions, also the hybrid lacks one line found in the parental species. Plants of the same species, but not the parental plants were used in this test.




12 common lines



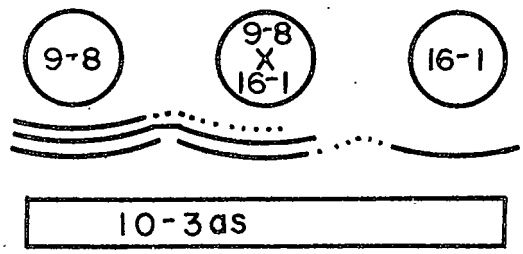

7 common lines

Fig. 4 Drawing of the serological plates showing the difference observed between two plants of A. intermedium and their supposed hybrid. Note the difference between the two plants of A. intermedium.

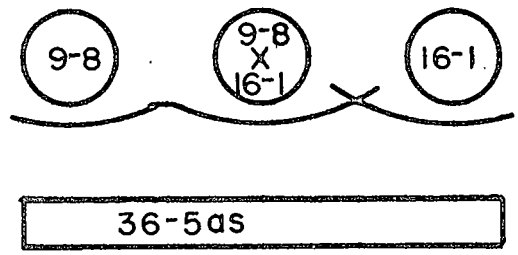
antigen capable of reacting with 10-3 antiserum which was not found in 44-2 and two antigens capable of reacting with 36-5 antiserum not found in 44-2. This represents a definite genetic difference between plants of the same species.

The suspected hybrid A. ciliatiflorum X A. cristatiforme and the seed parent A. ciliatiflorum each showed two precipitate lines that A. cristatiforme did not have when tested with antiserum 10-3. When tested with antiserum 36-5, the putative hybrid and A. ciliatiflorum showed one precipitate line not found in A. cristitiforme; and A. cristatiforme showed one precipitate line not found in the putative hybrid or A. ciliatiflorum. These different precipitate lines appear on the plate as crossed lines (Type II reaction) and are shown in Figure 5.

For the putative hybrid A. ciliatiflorum X A. cristatiforme the antigen sets are assigned A. ciliatiflorum $C_i$, A. cristatiforme $C_r$ and the putative hybrid $C_h$. In the universe $V_{10-3}$, $C_i \supset c_1, c_2$ and $C_r \supset c_1$. This is unexpected as 10-3 is also A. cristatiforme and $C_r$ should come close or equal to $V_{10-3}$. This is another indication of unexpected intra-specific variation. In the universe $V_{36-5}$, $C_i \supset c_1, c_2$ and $C_r \supset c_1, c_3$. $C_h \supset c_1, c_2$ in both $V_{10-3}$ and $V_{36-5}$. In this case $C_h = C_i$ and $C_h \neq C_i \cup C_r$. It is concluded that $C_h$ is not a hybrid.




10 common lines

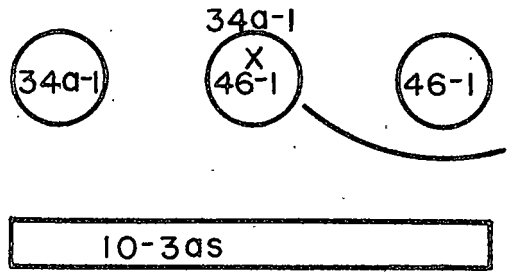



8 common lines

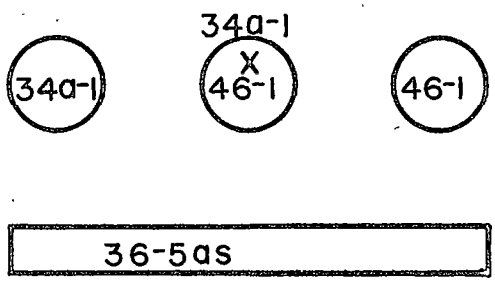
Fig. 5 Drawing of the serological plates showing the difference observed between A. ciliatiflorum, A. cristatiforme and their putative hybrid. Note the type II reaction shown between A. cristatiforme and the putative hybrid when tested against antisera from rabbit 36-5. The dotted lines indicate very weak reactions.

When the putative hybrid A. intermedium X A. junceum was tested with its supposed parents against antiserum 36-5, all lines were in common. The pollen parent, A. junceum showed a precipitate line that the putative hybrid and A. intermedium did not have when tested against antiserum 10-3 (Figure 6).

The putative hybrid A. intermedium X A. junceum is assigned the following set of symbols. A. intermedium $I_3$, A. junceum J and the putative hybrid IJ. In the universe $V_{10-3}$, $I_3 \supset j_1$ and $J \supset j_1, j_2$.




10 common lines




10 common lines

Fig. 6 Drawing of the serological plates showing the difference observed between A. intermedium, A. junceum and their putative hybrid.

In the universe $V_{36-5}$, $I_3 \supset j_1$ and $J \supset j_1$. The hybrid was found to contain the subset $j_1$ in the universe $V_{36-5}$ and $IJ \supset j_1$ in the universe $V_{10-3}$. The formula for the universe $V_{36-5}$ is:

$$IJ = I_3 = J.$$

The formula for the universe $V_{10-3}$ is:

$$IJ = I_3 \text{ and } IJ \neq I_3 \cup J.$$

It is concluded that IJ is not a hybrid. It does not contain all the antigens found in the supposed male parent.

The putative hybrid A. intermedium X A. elongatum and the seed parent A. intermedium showed a precipitate line not found in the pollen parent A. elongatum when tested against antiserum 10-3. When tested against antiserum 36-5, A. elongatum showed a precipitate line not found in either the hybrid or the seed parent (Figure 7).

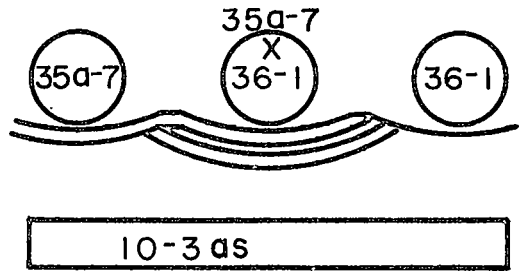
The putative hybrid A. intermedium X A. elongatum is assigned the following set of symbols. A. intermedium $I_4$, A. elongatum E and the putative hybrid IE. In the universe $V_{10-3}$, $I_4 \supset i_1$, $i_2$ and $E \supset i_1$. In the universe $V_{36-5}$, $I_4 \supset i_1$ and $E \supset i_1$, $i_3$. The hybrid was found to contain the subsets $i_1$, $i_2$ in the universe $V_{10-3}$ and $IE \supset i_1$ in the universe $V_{36-5}$. The formula for the universe $V_{10-3}$ is:

$$IE = I_4 \text{ and } IE = I_4 \cup E$$

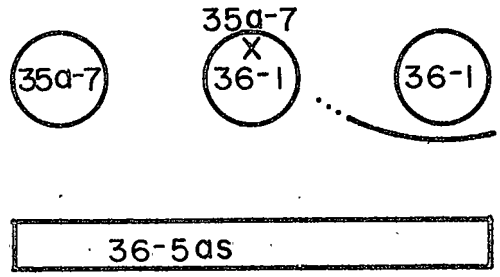
The formula for the universe $V_{36-5}$ is:

$$IE = I_4 \text{ and } IE \neq I_4 \cup E.$$

It is concluded that IE is not a hybrid. It does not contain all




9 common lines




8 common lines

Fig. 7 Drawing of the serological plates showing the difference observed between A. intermedium, A. elongatum and their putative hybrid.

the antigenic material found in the supposed male parent.

The putative hybrid A. spicatum (n-14) X A. pycnanthum was tested with its presumed parents and the supposed pollen parent A. pycnanthum showed two precipitate lines not in common with the seed parent or the supposed hybrid when tested against antiserum 10-3, and one precipitate line not in common with the seed parent or the putative hybrid when tested against antiserum 36-5 (Figure 8).

The putative hybrid A. spicatum X A. pycnanthum is assigned the following set of symbols. A. spicatum S, A. pycnanthum P and the putative hybrid SP. In the universe $V_{36-5}$, $S \supset s_1$ and $P \supset s_1, s_2$. The suspected hybrid was identical to S in both $V_{10-3}$ and $V_{36-5}$. The formula for both $V_{10-3}$ and $V_{36-5}$ becomes:

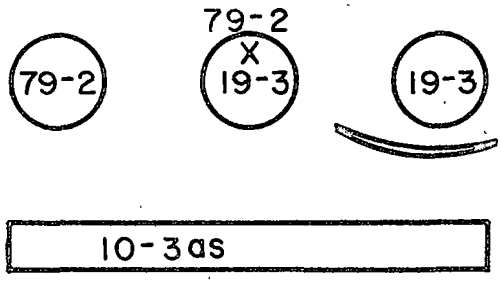
$$SP = S \text{ and } SP \neq S \cup P.$$

It is concluded that SP is not a hybrid. It does not contain all the antigenic material found in the supposed pollen parent.

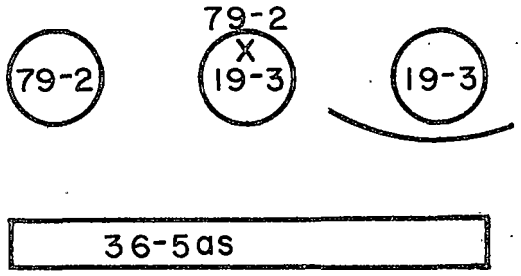
II. Serological Analysis of the B Genome

Individual plants of 11 species of Agropyron (Table IV) were tested against four different antisera (Table V) which had been absorbed or partially absorbed with antigenic material from A. spicatum (n=7). For these tests, S denotes the set of antigen-antibody responses with the diploid A. spicatum antigenic material. S', the complement to S, is all the rest of the possible reactions to a particular antiserum. This may be described by:

$$V_x = S \cup S' \text{ and } S' = V_x - S.$$




8 common lines




8 common lines

Fig. 8 Drawing of the serological plates showing the difference observed between A. spicatum (n=14), A. pycnanthum and their putative hybrid.

Table IV. A list of 11 species of *Agropyron* compared with the diploid *A. spicatum*.

| Species | MSU Field No. | Chromosome No. | Genome Formula (Author) |
|---|---|---|---|
| *A. arizonicum* | 2-8 | n = 14 | $S_4S_4B_4B_4$(Allderdice 1965) |
| *A. brachyphyllum* | 3-4 | n = 21 | $S_5S_5B_5B_5XX$*(Allderdice 1965) |
| *A. caninum* | 6-3 | n = 14 | $S_2S_2B_2B_2$(Allderdice 1965) |
| *A. ciliatiflorum* | 9-8 | n = 14 | ? |
| *A. dasystachyum* | (Collins) | n = 14 | SSXX (Dewey 1967) |
| *A. latiglume* | 48-1 | n = 14 | $S_2S_2B_2B_2$(Allderdice 1965) |
| *A. panormitanum* | 50a-1 | n = 14 | ? |
| *A. riparium* | 62a-4 | n = 14 | $S_3S_3B_3B_3$(Allderdice 1965)<br>SSXX(Dewey 1967) |
| *A. spicatum* | 79-2 | n = 14 | SSSS (Dewey 1967)<br>SSXX (Collins 1965) |
| *A. subsecundum* | 185 | n = 14 | $S_2S_2B_2B_2$(Allderdice 1965) |
| *A. trachycaulum* | 93-2 | n = 14 | $S_1S_1B_1B_1$(Allderdice 1965) |

* X indicates a genome of unknown origin

Table V. Antisera absorbed or partially absorbed with *A. spicatum* (n=7)

| Rabbit No. | Species Used to Induce the Production of Antibodies | Plant No. |
|---|---|---|
| 147-S | *A. dasystachyum* | 147-S |
| 10-3 | *A. cristatiforme* | 10-3 |
| 68-5 | *A. sibiricum* | 68-5 |
| 025 | *A. desertorum* | 30-10 |

This experiment was designed to test the possibility that the B genome found in A. trachycaulum, A. arizonicum, and A. caninum may be a modification of the S genome represented by the diploid A. spicatum as proposed by Schulz-Schaeffer and Jura (1967). By removing those antibodies that will react with antigenic material from A. spicatum (n=7) an estimate of the genes not present (or inactive), which will make a serological response, will be obtained. Each serological precipitate line observed on the plates may be described as:

$$S' \cap A = a_1 \text{ where } a_1 \text{ is a subset of } A.$$

Two antisera, 10-3 and 68-5 each had an antibody with a very high titer which along with the low titer of the antigenic material in A. spicatum (n=7) which would react with this antibody, resulted in a very low titer serum when the antibody was removed. This was due to excess dilution of the serum with the leaf extract. Partially absorbed antisera were used (Figure 9). To interpret the results using the partially absorbed serum, the number of lines with A. spicatum (n=7) was subtracted from the total number of lines observed.

In the absorption tests the following sets are designated; A. spicatum (n=7) S, A. spicatum (n=14) $S_4$, A. arizonicum A, A. brachyphyllum B, A. caninum $C_a$, A. ciliatiflorum $c_1$, A. latiglume L, A. dasystachyum D, A. panormitanum P, A. riparium R, A. subsecundum $S_u$ and A. trachycaulum T.

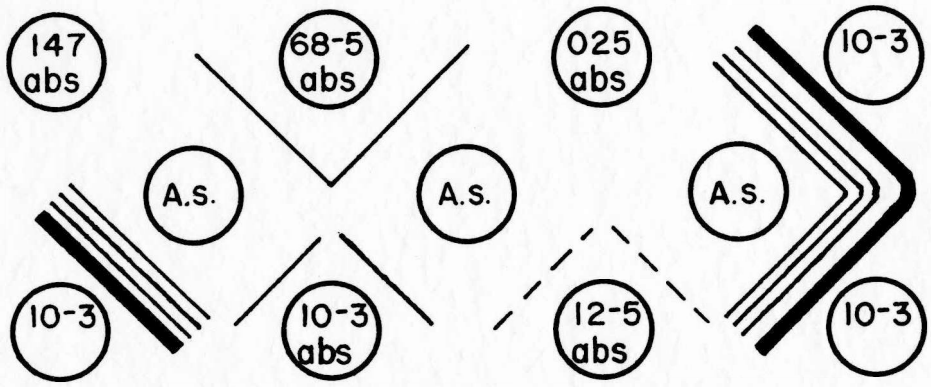



Fig. 9 Drawing of the serological plate showing the absorbed and partially absorbed antisera. Note the difference between the partially absorbed and unabsorbed 10-3 antisera. All antisera absorbed with A. spicatum (n=7).

In the universe $V_{147-s}$ (Figure 10) $S' = V_{147-s} - S$ and

$A \cap S' = \emptyset$, $B \cap S' = \emptyset$, $C_a \cap S' = \emptyset$, $C_i \cap S' = c_1$,

$D \cap S' = d_1$, $L \cap S' = \emptyset$, $P \cap S' = \emptyset$, $R \cap S' = c_2$,

$S_4 \cap S' = \emptyset$, $S_u \cap S' = d_1$ and $T \cap S' = \emptyset$.

The reaction of A. dasystachyum was not as expected. The antiserum 147-s was produced against antigenic material to a different collection of A. dasystachyum. Because $c_1$ and $c_2$ contain more lines than $d_1$ it is concluded by the same reasoning as used with A. cristatiforme that this is an example of intraspecific genetic differences among plants of A. dasystachyum. The subsets $c_1$ and $c_2$ may or may not be equivalent and may or may not contain $d_1$.

In the universe $V_{10-3}$, (Figure 12) $S' = V_{10-3} - S$ and

$A \cap S' = B \cap S' = C_a \cap S' = a_1$, $C_i \cap S' = a_2$,

$D \cap S' = L \cap S' = P \cap S' = S_4 \cap S' = S_u \cap S' = T \cap S' = \emptyset$,

and $R \cap S' = a_3$. The subsets $a_2$ and $a_3$ may or may not be equal and may or may not contain $a_1$.

In the universe $V_{68-5}$, (Figure 13) $S' = V_{68-5} - S$ and

$A \cap S' = C_a \cap S' = S_4 \cap S' = \emptyset$, $B \cap S' = b_1$,

$C_i \cap S' = b_2$, $D \cap S' = S_u \cap S' = b_3$, $L \cap S' = b_4$,

$R \cap S' = b_5$ and $T \cap S' = b_6$. The subset $b_4$ is a part of the subset $b_2$. The subsets $b_1$, $b_3$, $b_4$, and $b_5$ may be equivalent and, or, may be contained in the subset $b_2$.

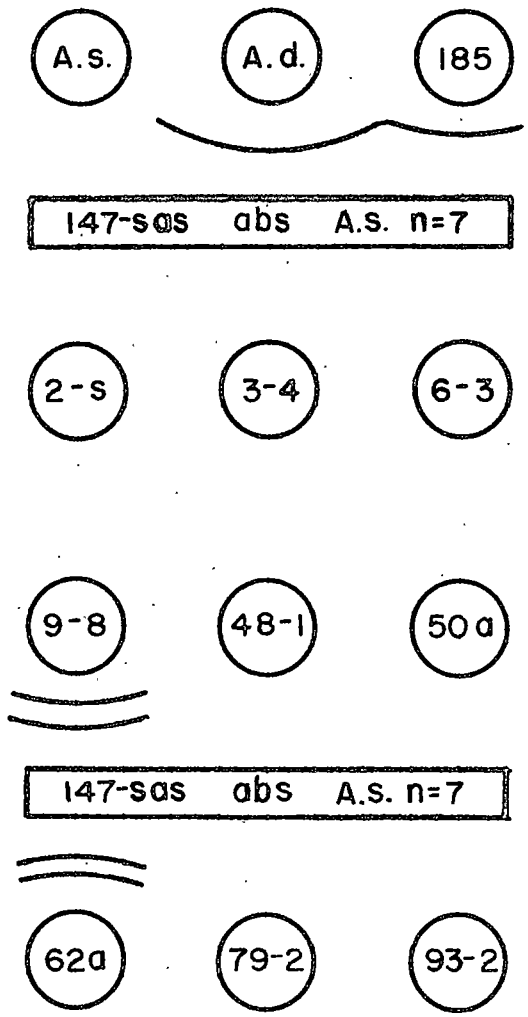



Fig. 10 Drawing of the serological plates showing 11 species of Agropyron tested with antisera 147-S absorbed with A. spicatum (n=7).

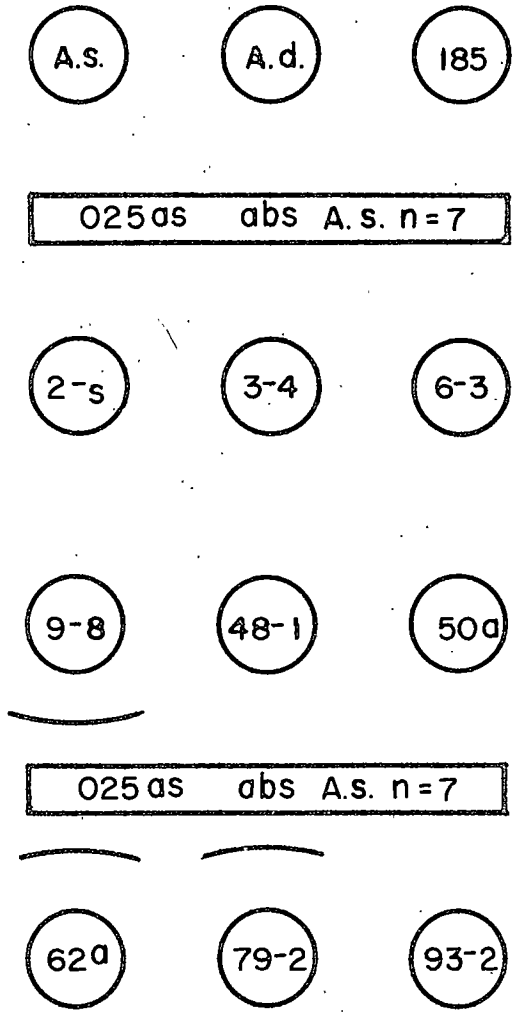



Fig. 11 Drawing of the serological plates showing 11 species of Agropyron tested with antisera 025 absorbed with A. spicatum (n=7).

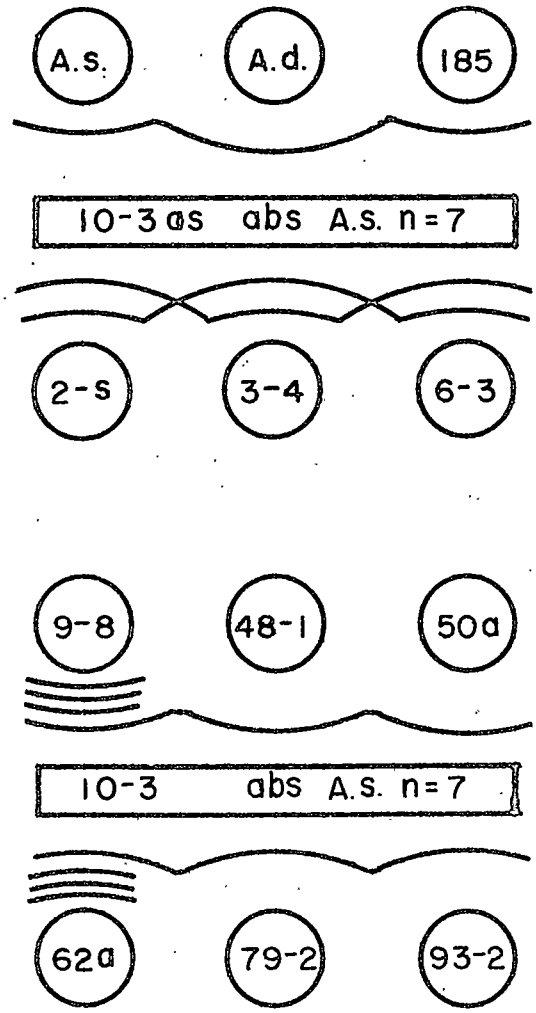



Fig. 12 Drawing of the serological plates showing 11 species of Agropyron tested with antisera 10-3 partially absorbed with A. spicatum (n=7).

In the universe $V_{025}$, (Figure 11) $S' = V_{025} - S$ and

$A \cap S' = B \cap S' = C_a \cap S' = D \cap S' = L \cap S' =$

$P \cap S' = T \cap S' = \emptyset$,

$C_1 \cap S' = d_1$, $R \cap S' = d_2$ and $S_4 \cap S' = d_3$.

The subsets $d_1$, $d_2$, and $d_3$ may or may not be equal. The lines representing $d_2$ and $d_3$ did not join or cross so an inference of similarity or difference between them may not be drawn.

Subset $d_3$ shows the only difference detected between A. spicatum (n=7) and A. spicatum (n= 14). Six plants had only one line in common when tested with all four absorbed antisera. These were A. arizonicum, A. caninum, A. latiglume, A. panormitanum, A. spicatum (n=14) and A. trachycaulum. Three plants of the species A. brachyphyllum, A. dasystachyum, and A. subsecundum each formed one line with two separate antisera. A. ciliatiflorum and A. riparium formed the greatest numbers of lines and both reacted with all four absorbed antisera. These data are summarized in Table VI.

III. Forced Selfing

To estimate the amount of homozygosity and heterozygosity present in the plants studied, an estimate of the mating system was made by determining seed set under conditions of forced selfing and open pollination. These data are presented in Table VII.

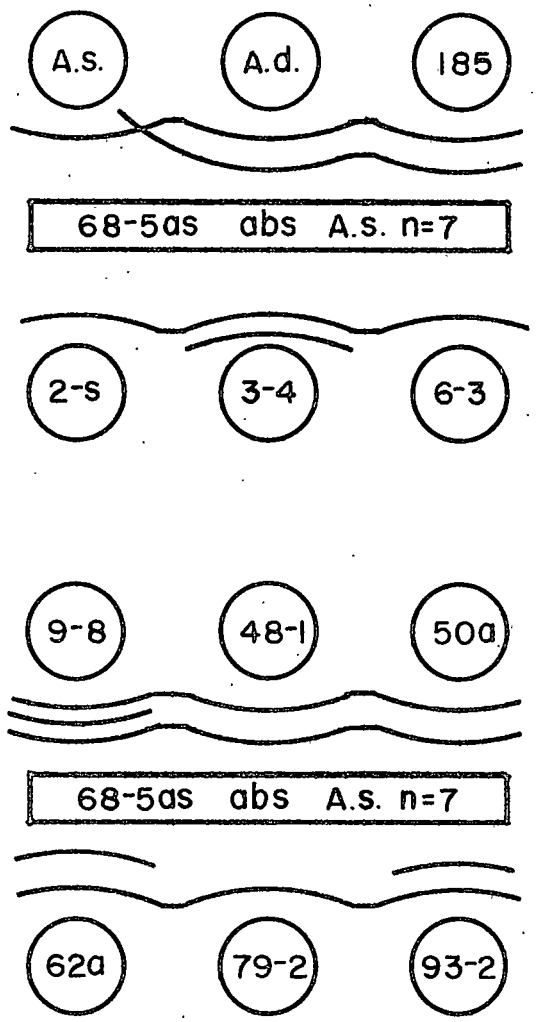



Fig. 13 Drawing of the serological plates showing 11 species of Agropyron tested with antisera 68-5 partially absorbed with A. spicatum (n=7).

Table VI. Net number of lines observed using antisera absorbed and partially absorbed with *A. spicatum* n=7

| Species | Plant No. | Antisera 147 | 025 | 10-3* | 68-5* | Difference Detected Min.-Max. |
|---|---|---|---|---|---|---|
| *A. spicatum* (n=7) (Belgrade) | | - | - | - | - | 0-0 |
| *A. arizonicum* | 2-8 | - | - | 1 | - | 1-1 |
| *A. brachyphyllum* | 3-4 | - | - | 1 | 1 | 1-2 |
| *A. caninum* | 6-3 | - | - | 1 | - | 1-1 |
| *A. ciliatiflorum* | 9-8 | 2 | 1 | 3 | 2 | 3-8 |
| *A. dasystachyum* | 147-S | 1 | - | - | 1 | 1-2 |
| *A. latiglume* | 48-1 | - | - | - | 1 | 1-1 |
| *A. panormitanum* | 50a-S | - | - | - | 1 | 1-1 |
| *A. riparium* | 62a-4 | 2 | 1 | 3 | 1 | 3-7 |
| *A. spicatum* n=14 | 79-2 | - | 1 | - | - | 1-1 |
| *A. subsecundum* | 185 | 1 | - | - | 1 | 1-2 |
| *A. trachycaulum* | 93-2 | - | - | - | 1 | 1-1 |

*One antigen removed from each reaction because of incomplete absorbtion of these antisera.

Table VII. Seed set of 10 strains of 9 *Agropyron* species after open-and self-pollination according to field observations during the summers of 1962, 1963, 1964, 1965 and 1966.

| Species | Plant No. And Year | Open-Pollination Florets | Open-Pollination Seed | Open-Pollination Percent | Self-Pollination Florets | Self-Pollination Seed | Self-Pollination Percent | Percent Self-Fertile |
|---|---|---|---|---|---|---|---|---|
| *A. arizonicum* | 2-5(63) | 301 | 244 | 81.1 | 227 | 175 | 77.9 | |
| | 2-2(65) | | | | 160 | 49 | 30.6 | |
| | 2-6(66) | 476 | 348 | 73.1 | 169 | 144 | 84.6 | |
| | Total | 777 | 592 | 76.3 | 556 | 368 | 66.2 | 86.9 |
| *A. brachyphyllum* | 3-2(63) | 300 | 225 | 75.0 | 93 | 31 | 54.8 | |
| | 3-6(66) | 301 | 224 | 74.4 | 298 | 240 | 80.5 | |
| | | 601 | 449 | 74.8 | 391 | 271 | 69.4 | 92.8 |
| *A. caninum* | 6-1(63) | | | | 61 | 51 | 83.6 | |
| | 6-4(63) | | | | 105 | 85 | 80.9 | |
| | 6-4(63) | 300 | 205 | 68.3 | 230 | 113 | 49.1* | |
| | 6-9(66) | 204 | 161 | 78.9 | 253 | 200 | 79.0 | |
| | | 504 | 366 | 72.6 | 649 | 449 | 69.2 | 95.4 |
| *A. ciliatiflorum* | 9-3(63) | 300 | 257 | 85.7 | 189 | 165 | 87.3 | 100 |
| *A. latiglume* | 48-3(63) | 300 | 257 | 85.7 | 214 | 102 | 47.7 | |
| | 48-3(66) | 410 | 253 | 61.7 | 199 | 98 | 49.2 | |
| | | 710 | 510 | 71.7 | 413 | 200 | 48.4 | 57.7 |
| *A. panormitanum* | 50a-2(63) | 300 | 235 | 78.3 | 116 | 96 | 82.8 | |
| | 50a-6(66) | 562 | 358 | 63.7 | 616 | 460 | 74.7 | |
| | | 862 | 593 | 68.8 | 732 | 556 | 75.9 | 100 |

Table VII. (cont'd)

| Species | Plant No. And Year | Open-Pollination Florets | Open-Pollination Seed | Open-Pollination Percent | Self-Pollination Florets | Self-Pollination Seed | Self-Pollination Percent | Percent Self-Fertile |
|---|---|---|---|---|---|---|---|---|
| *A. riparium* | 62a-4(62) | 500 | 21 | 4.2 | | | | |
| | 62a-1(65) | | | | 21 | 0 | 0.0 | |
| | 62a-2(65) | | | | 108 | 1 | 0.9 | |
| | 62a-3(65) | | | | 808 | 0 | 0.0 | |
| | | 500 | 21 | 4.2 | 937 | 1 | 0.1 | 2.4 |
| *A. spicatum*(n=14) | 72-2(66) | 255 | 61 | 23.9 | 451 | 23 | 5.1 | 21.3 |
| *A. spicatum*(n= 7) (5 plants) | Belgrade(66) | 606 | 3 | 0.5 | 577 | 0 | 0.0 | ---- |
| *A. trachycaulum* | 93-4(63) | 500 | 258 | 51.6 | 69 | 26 | 37.7* | |
| | 93-5(66) | 413 | 153 | 37.0 | 655 | 148 | 22.6 | |
| | | 913 | 411 | 45.0 | 724 | 174 | 24.0 | 53.4 |

* Low seed set due to shattering prior to harvest

$$\text{Percent self-fertile} = \frac{\text{percent self-pollination}}{\text{percent open-pollination}}$$

Seed set studies showed that some species of Agropyron are capable of high seed set under conditions of forced self-pollenation (Table VII). These include A. arizonicum, A. brachyphyllum, A. caninum, A. ciliatiflorum, A. latiglume, A. panormitanum and A. trachycaulum. Most of the plants tested showed a high percentage seed set under conditions of forced self-pollenation. Exceptions were the tetraploids, A. riparium and A. spicatum which had very low seed set under forced selfing and field conditions and the native diploid A. spicatum which set no seed the summer of 1967.

The nine species in the selfing studies could be divided into three groups: 1) those which are capable of near 100% self-fertilization, these are A. arizonicum, A. brachyphyllum, A. caninum, A. ciliatiflorum and A. panormitanum; 2) those capable of approximately 50% self-fertilization which are A. latiglume and A. trachycaulum and 3) those with very low seed set, 5% or less, which are A. riparium and A. spicatum (n=14). Under conditions of open pollination A. riparium and A. spicatum (n=14) had a maximum seed set of 25%. The diploid A. spicatum set almost no seed under native conditions during 1966. This was thought to be due to unknown environmental conditions such as humidity which may have inhibited pollen viability or distribution.

The mating system appears to be predominately selfing with some outcrossing. A. riparium and A. spicatum (n=14) are exceptions which appear to be primarily cross-pollinated species.

IV. Technique

A. Tissue Differences

To test the possibility that one portion of a plant might contain antigenic material not found in another portion of the same plant, sap was expressed from the culms and spikes of plant 10-3 (Creel et al. 1965) and compared with leaf extract from the same plant (Figure 14). It was found that the leaf material (R-Ag) contained material not found in the culms and spikes (X-Ag). The R-Ag (leaf) produced precipitate bands not produced by the X-Ag (stem and culm antigenic material). These data are

shown in Figure 14 which also shows the differences between the two antisera 10-3 and 010. Antiserum 009 reacted similarly to antiserum 010 but was not as strong.

B. Animal Differences

To determine the response of unrelated rabbits to the same antigenic material, unrelated rabbits 009 and 010 were treated identically with antigenic material from plant 10-3. These antisera were plated with antiserum from rabbit 10-3 as shown in Figure 15B. These data show nonidentical response to the antigenic material. Three possible explanations for the different response are: 1) different rabbit genotypes respond differently to the same antigenic material; 2) prolonged immunization of rabbit 10-3 resulted in a serum of high fidelity and 3) the prior immune history of the rabbits. All of these explanations may be applicable to the present situation. The former may be the most valid in explaining the titer difference between rabbits 009 and 010 while the second appears to be valid because of the prolonged immunization of rabbit 10-3. The immune history of the rabbits was unknown.

An experiment was designed to test the possibility that the genotype of the rabbits had an effect on the ability to produce antibodies against plant antigenic material. Four rabbits (three male and one female) were obtained from the same litter (025, 026, 027 and 028), and treated identically using antigenic

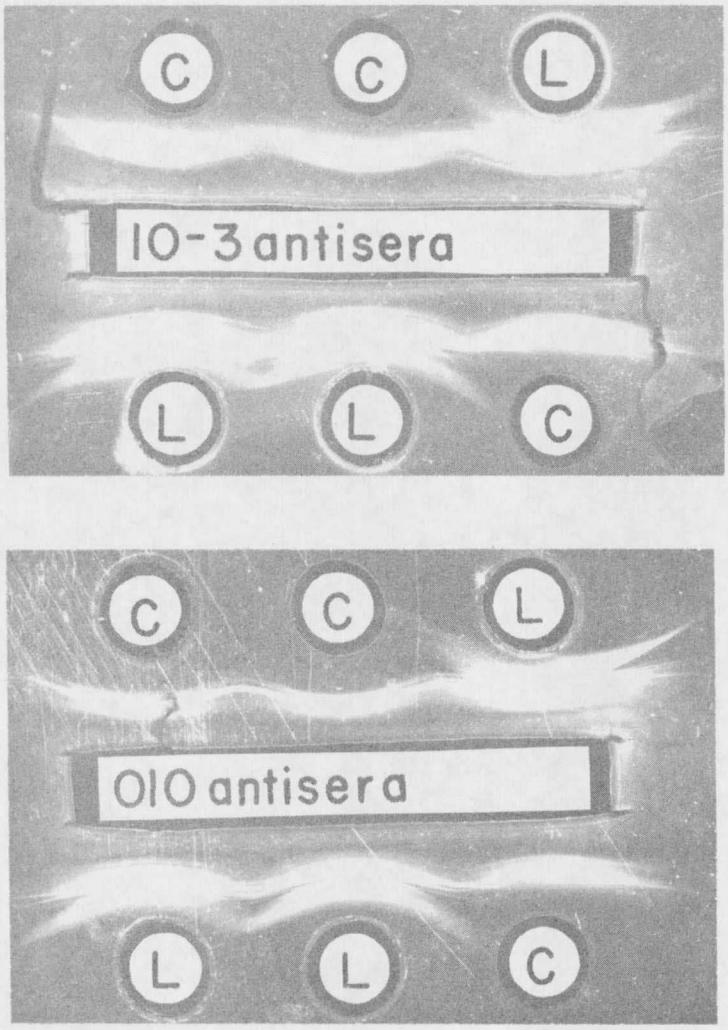



Fig. 14 Photographs of antigen-antibody reactions observed when leaf antigenic material and culm and spike antigenic material from plant 10-3 were tested with antisera 10-3 and 010. L = leaf antigenic material, C = culm and spike antigenic material.

material from plant 30-10, A. desertorum. All four resulting antisera were reacted with the reference antigen on the same plate and compared. No titer or line differences were detected (Figure 15A). The expected genetic correlation between litter mates is .50 and these rabbits probably had a similar immune history because they were litter mates. These data indicate that it may be difficult to duplicate an antiserum at some future time. An inbred strain of rabbits selected for their antibody production might help in this respect.

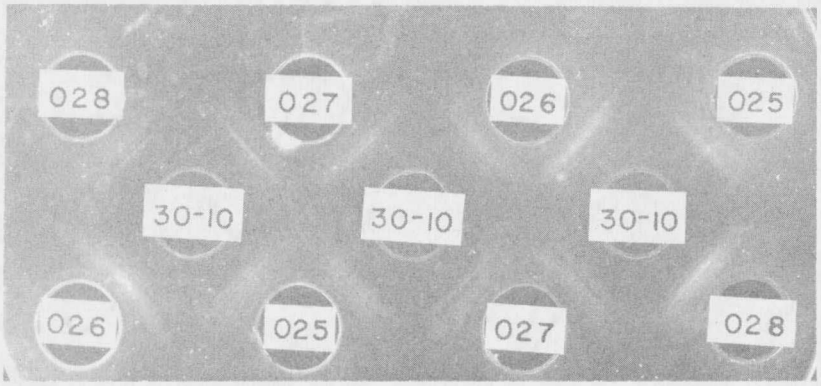



Fig. 15A Photograph of the reaction of four antisera produced against plant 30-10. No differences would be detected with these antisera which were produced in litter mates.

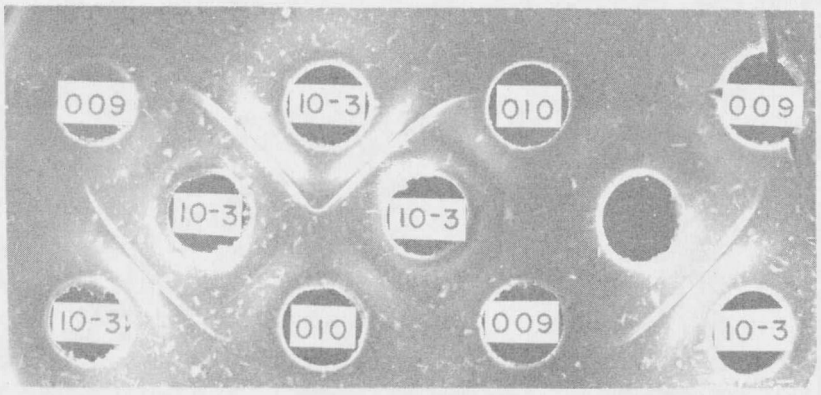



Fig. 15B Photograph of the reactions of three antisera produced against plant 10-3. Note the difference between sera produced by rabbits 009 and 010 when the rabbits were given identical treatments. Rabbit 10-3 produced this antiserum after a prolonged immunization schedule.

# DISCUSSION

## I. General

The two components of a serological reaction are the antigen and the antibody. Antigens are predominately proteins with a molecular weight between 34,500 and 5,000,000 capable of inducing the formation of antibodies in the host. Some polysaccharides and nucleic acids may have antigenic properties (Kwapinski 1965). Recent concepts of protein synthesis (Watson 1962) allow the assumption that antigens are gene products. Thus, when differences in antigenic response are considered, genetic differences are implied. Antibody synthesis takes place in plasma cells and lymphocytes of the spleen and lymph nodes (White et al. 1955) and are highly specific for the production of antibodies against a specific antigen (Coons 1958). "It is assumed that these cells have unique sequences of nucleotides in segments of the chromosomal deoxyribonucleic acid, which are genes for the globulin synthesis" (Kwapinski 1965). Thus, antibodies are controlled genetically.

Antigenic material from individual plants has a characteristic antigen-antibody pattern when tested against a particular antiserum. Landsteiner (1945) defines the specificity of antibodies as X "a disproportional action of a number of similar agents on a variety of related substrates". "The specificity of antibodies provides a powerful tool for the detection, identification and differentiation of antigens" (Kwapinski 1965). This specificity allows for comparisons among plants within a species, comparisons among plants

between species and parent - hybrid comparisons.

II. Hybrid and Putative Hybrid Comparisons

Three hybrids analyzed by Allderdice (1965) contained all of the antigens of both parents when tested with antisera 10-3 and 36-5. All lines were found to be in common between the hybrid and its two parents.

The hybrid A. brachyphyllum X A. riparium (Allderdice 1965) was tested against plants of the same collection of A. brachyphyllum and a plant from a different collection of A. riparium. The hybrid did not contain all of the antigens found in the parental species. This may be a case of intraspecific variation among plants of A. brachyphyllum and A. riparium. If this is a case of intraspecific variation then the exact parents should be used in these tests. Another possible explanation would be that both parents were heterozygous for the allele responsible for the production of the antigen and the hybrid lacks the ability to produce the antigen because it is homozygous recessive for the allele under consideration.

The assumption that antigen-antibody spectra are species specific has been implied by earlier plant serological work (Lester 1964, Creel 1964 and Fairbrothers 1966). If this assumption holds in Agropyron then differences among plants within a species are not expected. The two parental plants of A. intermedium which were tested against their putative hybrid did not respond identically.

If the above assumption is hypothesized then no differences between plants of the same species are expected. Plant 34a-1 contained one antigen capable of reacting with antiserum 10-3 and two antigens capable of reacting with antiserum 36-5 not found in plant 44-2. These represent definite genetic differences between plants of the same species. Therefore, the assumption that all antigen-antibody stectra are species specific must be discarded. This is not to imply that all relations based upon antigen-antibody relations should be discarded but rather that caution should be observed in interpretation.

The seed parent and the supposed hybrid of the A. intermedium (34a-1) X A. intermedium (44-2) attempted cross were serologically different than the supposed pollen parent. More differences were found between the two plants of A. intermedium than Creel (1964) found between the annual A. hirsutum and the perennial A. spicatum (n=14). Many workers consider A. hirsutum to belong to the genus Eremopyrum (Nevski 1934, Sarkar 1958, Bowden 1962, Sakamoto and Muramatsu 1965). If the correct classification for the taxon "A. hirsutum" is Eremopyrum hirsitum Nevski then a greater difference was detected among plants of the same species than Creel (1964) was able to demonstrate among plants from two genera.

The putative hybrid A. ciliatiflorum X A. cristatiforme and A. ciliatiflorum showed more serological lines with antiserum 10-3 than did A. cristitaforme (16-1). Intra-specific variation is

indicated because 10-3 is also a collection of A. cristatiforme and 16-1 is expected to show the maximum number of serological lines with 10-3 antiserum.

The putative hybrids A. intermedium X A. junceum, A. intermedium X A. elongatum and A. spicatum X A. pycnanthum did not contain all of the antigenic material of the supposed pollen parent and it was concluded that they were not hybrids.

III. Serological Analysis of the B Genome

Stebbins and Singh (1950), Stebbins and Pun (1953) and Stebbins and Vaarama (1954) have shown that the genome represented by the diploid Agropyron spicatum and A. inerme is widespread in the following genera: Agropyron, Sitanion and Elymus. There is a possibility that the diploid A. spicatum and A. inerme are the diploid level of a pivotal genome. This genome has been designated genome S by Schulz-Schaeffer and Allderdice (1966).

Lorenz and Schulz-Schaeffer (1964), using paper chromatographic techniques, showed A. spicatum (n=7) to be quite closely related to A. caninum, A. subsecundum and A. trachycaulum.

Individual plants of 11 species of Agropyron (Table IV) were tested against antisera which had been absorbed with antigenic material from diploid A. spicatum. Tetraploid A. spicatum showed one antigen-antibody reaction with antiserum 025. This was the only serological difference detected between the diploid and tetraploid forms of A. spicatum. Dewey (1967) considers tetraploid A. spicatum

to be an autopolyploid with the genome formula SSSS. Collins (1965) gave the tetraploid A. spicatum the genome formula SSXX. Both based their information on the meiotic behavior of the plants studied. If Collins (1965) and Dewey (1967) are considered together, Collins did not show that the "X" genome he proposed was very much different from the "S" genome of Dewey. Because the small amount of serological difference found between the diploid and tetraploid A. spicatum is less than the amount of plant to plant variation found, it would appear that the X genome of Collins is very likely a slightly modified S genome. Perhaps the tetraploid has been formed independently in several localities as an autopolyploid, then the genome formula might be written as $SSS_xS_x$ where x denotes an unknown amount of variable change found in the differing populations of tetraploid A. spicatum.

Five plants in addition to the diploid A. spicatum had only one line in common when tested with all four absorbed antisera (Table VI). These were A. arizonicum, A. caninum, A. latiglume, A. panormitanum and A. trachycaulum. Four of these species have been shown to contain the SSBB genome combination. These are A. arizonicum, A. caninum, A. latiglume and A. trachycaulum. Three plants of the species A. brachyphyllum, A. dasystachyum and A. subsecundum each formed one line with two separate antisera. These three species also contain the SSBB genome complex. A. ciliatiflorum and A. riparium formed the greatest numbers of lines

and both reacted with all four absorbed antisera. A. riparium also has the genome formula SSBB while A. ciliatiflorum has an unknown genome constitution. These data suggest that the B genome found in A. riparium is considerably different from the B genome found in A. arizonicum, A. caninum and A. latiglume. This suggests that the B genome has become differentiated in the past and is considerably different in the various species in which it occurs. The karyogram of A. arizonicum shown by Schulz-Schaeffer and Jura (1967) shows two type "F" satellite chromosomes. If this type "F" satellite chromosome is an indicator for the S genome then the A. arizonicum B genome is partly homologous with the S genome. If the B genome is a modified S genome in A. arizonicum then this leads to the conclusion that the B genome in other species may be derived from the S genome.

It appears that the S genome may act as a pivotal genome buffering differences between the tetraploid species of Agropyron containing this genome. A pivotal genome would allow for the differentiation of any accompanying genome such as the B genome. With occasional crossing between tetraploids, the S genome buffers the hybrid and may maintain some fertility. This would allow for backcrossing, with introgression and perhaps cryptic structural hybridity. Stebbins (1950) points out that structural hybridity may result in speciation.

If there has been introgression and resultant cryptic struc-

Table VIII. Difference indexes (after Creel 1964) of 9 species of *Agropyron* containing the S genome.

| Species | Plant # | *A. arizonicum* | *A. brachyphyllum* | *A. ciliatiflorum* | *A. dasystachyum* | *A. latiglume* | *A. panormitanum* | *A. riparium* | *A. spicatum* | *A. trachycaulum* |
|---|---|---|---|---|---|---|---|---|---|---|
| *A. arizonicum* | 2-8 | 0 | .16 | .24 | .22 | .20 | .20 | .14 | .22 | .18 |
| *A. brachyphyllum* | 3-2 | | 0 | .08 | .10 | .08 | .16 | .06 | .10 | .10 |
| *A. ciliatiflorum* | 9-9 | | | 0 | .10 | .08 | .16 | .10 | .10 | .10 |
| *A. dasystachyum* | 147-S | | | | 0 | .14 | .10 | .16 | .08 | .20 |
| *A. latiglume* | 47-3 | | | | | 0 | .20 | .06 | .14 | .14 |
| *A. panormitanum* | 50a-7 | | | | | | 0 | .22 | .10 | .18 |
| *A. riparium* | 62a-2 | | | | | | | 0 | .16 | .08 |
| *A. spicatum* | 75-3 | | | | | | | | 0 | .12 |
| *A. trachycaulum* | 93-4 | | | | | | | | | 0 |

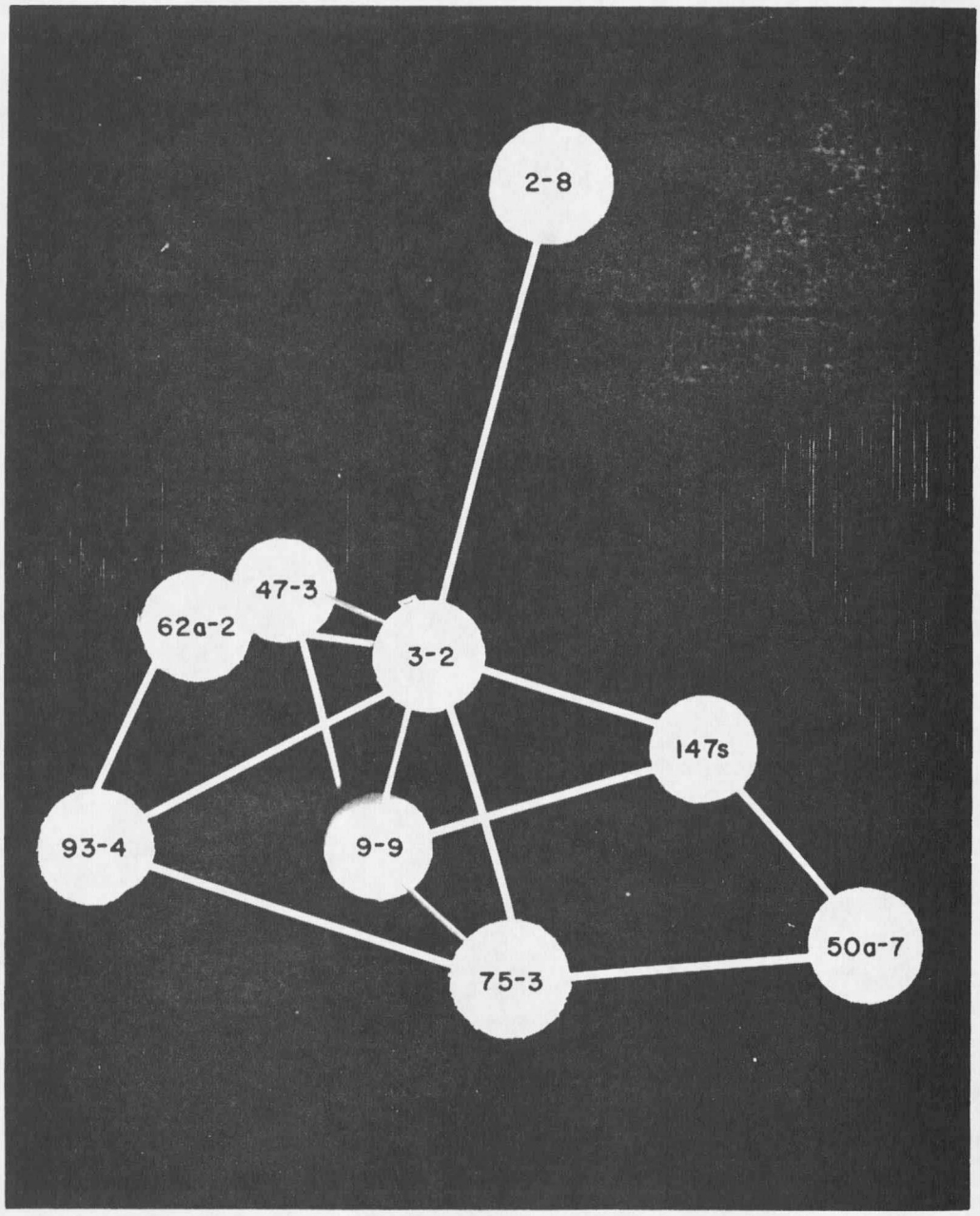



Fig. 16 Model of nine species of Agropyron using data of Creel (1964). Numbers refer to plant numbers listed in Table VIII.

tural hybridity in the past, then we can expect to find all stages of transition from both the purely autotetraploid, to segmental-allotetraploid, to the purely allotetraploid. Thus genome formulae, such as SSSS (autotetraploid), $SSS_BS_B$ (segmental-allotetraploid with more of the S than the B genome), $SSB_SB_S$ (segmental-allotetraploid with more of the B than the S genome), and SSBB (allotetraploid) are expected. It appears from the serological data that the A. spicatum (n=14) is an autotetraploid (Dewey 1967) varying to a segmental allotetraploid with a genome formula of $SSS_xS_x$. A. arizonicum may be a segmental allotetraploid with a genome formula of $SSS_BS_B$. Schulz-Schaeffer and Jura (1967) have shown two type F satellite chromosomes in the karyotype of A. arizonicum which tends to support this argument if the F type satellite chromosome is an indicator for the S genome as proposed by Schulz-Schaeffer and Jurasits (1962). Because of the large serological difference detected between A. riparium and the diploid A. spicatum, A. riparium may be an allotetraploid with the genome formula SSBB.

These genome formulae may be more accurate than those based only on cytological data because chromosomes which appear to be identical may have come from different genomes. Again, cryptic structural hybridity (Stebbins 1950) may not be distinguished in hybrid analysis or karyotype analysis. Cytological, morphological and serological data are each insufficient to determine the evolutionary structure of a taxon such as Agropyron. The taxonomy of

Agropyron must consider all available information in order to arrive at the most natural classification possible. It is proposed that serological analysis may play an important part in a biosystematic program but should not be considered as superior or inferior to other systematic data.

Data from the "four-way test" of Creel (1964) are presented in Table VIII. A model using these data was reconstructed and is shown in Figure 16. These data are presented as the "difference index" between each species. This difference Index, DI, was computed by summing the difference in number of lines found between each species as compared with four different antisera, and divided by the total number of lines found with the sum of the reference reactions of the four antisera.

$$DI = \frac{\sum V_n(X\text{-}Re_1 - X - Re_2)}{\sum V_n(R\text{-}Re_n)}$$

Based on the data presented by Creel eight of the nine species form a cluster around A. brachyphyllum and A. ciliatiflorum. A. arizonicum was distinct from the other eight species.

The present study does not agree with this model. A. cilialiflorum and A. riparium formed seven and eight precipitate lines respectively when tested with the four absorbed antisera. Six species, including A. arizonicum, were shown to contain only one antigen which would react with the four absorbed antisera. Three species formed two precipitate lines.

Creel's method measures minimum differences while absorbed antisera measures maximum differences in respect to four antisera.

Zohary (1965) has shown a combination of compound amphidiploidy, buffering pivotal genomes, and a predominance of self-pollination in the genus Aegilops. He speculated that the polyploid complexes of Agropyron contained a similar genetic system and that similar systems based on common genomes might be found to occur in other polyploid complexes.

III. Forced Selfing

The mating system in the Agropyron studied appears to be predominately selfing with some outcrossing. A. riparium and A. spicatum (n=14) are exceptions which appear to be primarily cross-pollinated species.

Self-fertilized species are expected to be largely homozygous while predominately outcrossing species should be more heterozygous. Stebbins (1950) states "...in predominantly self-fertilized species the ability of the rare heterozygous interracial hybrids to produce many new gene recombinations is of...high selective value as a means of adapting the species to new conditions. In these species...fitness and constancy are secured entirely through self-pollination and homozygosity, while...flexibility and adaption to new situations consists of short periods of great heterozygosity, following interracial hybridization".

Using an obligate self-fertilizing species, intraspecific differences in serological response could be investigated. It would be theoretically possible to obtain stocks homozygous for a particular marker and complementary stocks homozygous for an alternative allele. Once the stocks have been obtained, the inheritance of the serological marker can be established by appropriate mating techniques.

V. Technique

The data shown in Figure 14 indicate that identical plant parts should be used for serological comparisons. Leaves from plant 10-3 contain antigenic material not found in culms and spikes from the same plant.

Unrelated rabbits 009 and 010 which were treated identically with antigenic material from plant 10-3. Antisera from these rabbits was compared with antiserum from rabbit 10-3. The non-identical response may be due to three possible explanations: 1) different rabbit genotypes respond differently to the same antigenic material; 2) prolonged immunization of rabbit 10-3 resulted in a serum of high fidelity; and 3) the prior immune history of the rabbits. The immune history of the rabbits was unknown.

Moritz (1960, 1964) developed antisera of "high fidelity" by injections over a prolonged period of time. Some of Moritz's rabbits were over three years old and in good condition. Moritz suggests that prolonged immunization may result in antisera capable of reacting with more of the different antigenic materials in the

reference reaction. Other workers (Leone 1952, Kwapinski 1965, Boyden 1960), in zoological serology, disagree with prolonged immunization schedules. Rabbit 10-3 was immunized for over one year. Rabbits 009 and 010 were kept only long enough (three months) to develop sera of useable strength. The differences between rabbit 10-3 and rabbits 009 and 010 may be due to either genetic difference or to the prolonged immunization of rabbit 10-3.

SUMMARY

Data are presented which illustrate the complexity of the interaction between two genetic systems. Serologically four kinds of differences were found: 1) rabbit to rabbit differences illustrated by rabbits 009, 010 and 10-3 which did not respond the same to antigenic material from plant 10-3; 2) tissue to tissue differences illustrated by the comparison of the X-Re of antigenic material from culms and spikes of plant 10-3 with the R-Re of leaf antigenic material from plant 10-3; 3) plant to plant differences within a species illustrated by the A. intermedium intraspecific putative hybrid, the A. cristatiforme (16-1) X-Re with antiserum from rabbit 10-3 and the A. dasystachyum X-Re with antiserum from rabbit 147-s and 4) species to species difference similar to those reported by Creel (1964).

Set notation is developed as a tool for describing and interpreting antigen-antibody precipitate lines in double-diffusion gel plates. Based on serological evidence four of the five putative hybrids studied are apparently selfs rather than hybrids. One putative hybrid could not be distinguished as either a self or a hybrid by serological means.

A. spicatum (n=14) is considered a variable autotetraploid with a genome formulae of SSSS to $SSS_xS_x$, where x is a varying amount of change in the different populations of A. spicatum. A. arizonicum is considered to be a segmental allotetraploid and is given the genome formula of $SSS_BS_B$ based upon serological data and supported by cytogenetic information.

Cryptic structural hybridity may not be distinguished in hybrid analysis or karyotype analysis. Morphological information has not shown the relationships of the Agropyron. Serological information may be an important source of information in a biosystematic program but should not be considered as either superior or inferior to other systematic data.

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