*NEW TECHNIQUES IN ANALYSIS OF COMPLEX NATURAL HYBRIDIZATION**

By Ralph E. Alston and B. L. Turner

DEPARTMENT OF BOTANY AND THE PLANT RESEARCH INSTITUTE, UNIVERSITY OF TEXAS

Communicated by Wilson S. Stone, December 21, 1961

There are numerous examples of natural hybridization among plants, in some cases involving quite dissimilar species. Formal study of such hybridizing populations usually involves construction of a hybrid index of morphological characters. Since many morphological characters are qualitative and complex in their modes of inheritance, analysis of the dynamics of hybridization by use of morphological hybrid indices cannot be precise. If three or more species are involved in complex natural hybridization, morphological criteria are likely to be wholly inadequate, and an analysis of gene flow is impossible. This type of problem is encountered in the legume genus *Baptisia* of North America. *Baptisia* is exceptional in the degree of morphological difference between species which nevertheless may hybridize when they occur together. Among the more than 20 distinct *Baptisia* species, numerous cases of hybridization have been reported. In Texas, it is possible that complex hybridization involving four species in a single population will be discovered, since one population including all four species has already been located, and we have proved independently that in various locations hybrids between any two of the four species occur.[1] This report describes new methods which may be applied to analyses of either simple or complex hybridizations of *Baptisia* species and which may provide information about such populations not attainable by other methods now in use.

Recently, Turner and Alston[2] demonstrated, chromatographically, recombination of species-specific substances of *B. laevicaulis* and *B. viridis* in putative hybrids thus proving natural hybridization in that instance. Larisey[3] reported hybridization between *B. leucantha* and *B. viridis* near Beaumont, Texas. A third species, *B. laevicualis*, was also present in the area, but it was concluded that *B. laevicaulis* was not involved in the hybridization. These conclusions were based on morphological characters. We have since discovered that a fourth species, *B. nuttalliana*, is present in the area and have located one small population with members of all four species occurring together. By combined morphological and chromatographic data, we have now located and defined hybrids between any two of the four species involved although certain combinations are much more frequent than others.

In the occasional large populations of intermingled *B. laevicaulis*, *B. leucantha*, and *B. viridis*, a small number (perhaps 5 per cent) of apparently several types of "hybrids" are nearly always present. The three species are delimited by such striking morphological differences that hybrids are readily distinguished. Yet, the presence of three different species, potentially hybridizing and "back-crossing" independently (pure species are thought to self-pollinate infrequently, and self-sterility is suspected), provides a rather complex situation. It is, therefore, a difficult matter to study population dynamics by means of even a painstaking morphological analysis. In the tri-hybrid situation described above, putative hybrids of *B. leucantha* and *B. laevicaulis* were rare (and then highly conjectural). An

important question is that of whether the third species, *B. viridis* may act as a bridge for gene exchange between the other two. Related questions concerning the preferred hybridization patterns, degree of fertility of particular F_1 types, and possible selection in backcrossing are all relevant to an understanding of the evolutionary past and future of the population. In the work to be described, we have analyzed certain populations both morphologically and chromatographically. The chromatographic analysis provides several advantages over the morphological analysis and may represent a means of supplying objective data pertinent to certain of the questions raised above.

Experimental.—All plants used in this study were collected in the field in flower. The individuals from pure populations, serving as standard types for the morphological and chromatographic analyses of hybridizing populations, were obtained from areas where no other species of *Baptisia* were seen. Only ten individuals from each species were used to produce the composite species-representative chromatograms of Figures 1–3, but we have examined numerous other individuals from various populations, and the validity of the patterns is definitely established. Additional individual plants of each species as well as additional *B. viridis* × *B. leucantha* hybrids from several other populations are plotted in Figure 7. These last were analyzed to provide supplementary data on possible backcrossing, discussed below.

The tri-hybrid population consisted of upwards of 1,000 individuals with the three species nearly equally represented and distributed semirandomly (i.e., some relative-density population gradients for the species involved could be recognized). Approximately 5% of the population was estimated to be composed of suspected hybrids or hybrid derivatives. We selected mostly hybrid types with only a few pure types of each of the three species. The *B. viridis* × *B. leucantha* supplementary hybrids mentioned in the paragraph above were selected following screening of mass collections from hybridizing populations by one-dimensional chromatography to disclose hybrids.

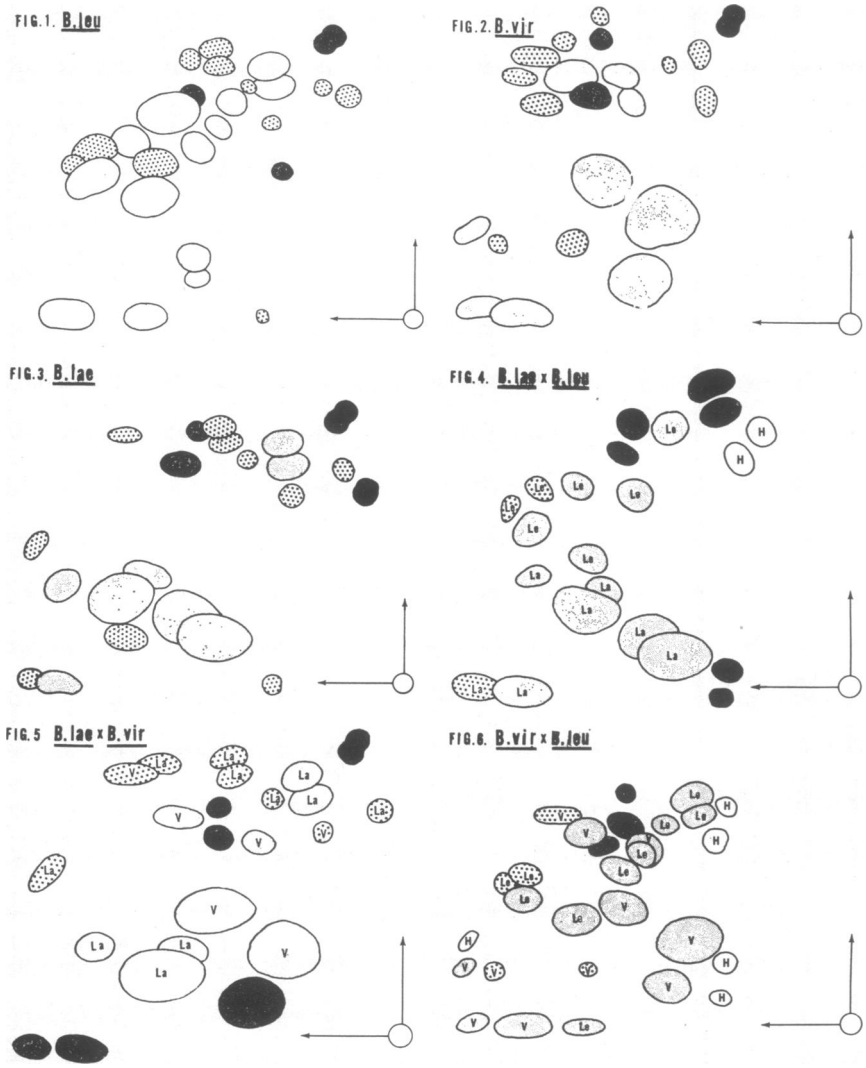
A morphological tri-hybrid index, utilizing a total of 20 carefully selected characters, was designed to yield a percentage value indicative of the genetic composition of the plant in question. A hybrid of A × B backcrossed to C would thus be expected to key ±25% A, ±25% B, and ±50% C in the tri-hybrid index. Details of the morphological tri-hybrid index will be published elsewhere, and only the final percentage values are included here (Table 1).

The chromatographic procedure was relatively simple once an appropriate pair of solvents was selected. The chief problem in selection of the solvents was to minimize overlapping of the various species-specific components of the three species. We have succeeded in eliminating almost all significant overlapping by use of the following solvents in the order listed: (1) tertiary butanol: acetic acid:water (3:1:1 v/v), and (2) acetic acid:water (15:85 v/v).

Plants were pressed and dried under standard conditions (45–50°C for 24 to 48 hr) and stored in light-tight herbarium cases. For chromatography, 6 to 12 mature leaves were extracted in 5 ml of 1% HCl in methanol in darkness for 24 hr. One hundred applications of the extract were applied directly to $18^1/_4'' \times 22^1/_2''$ sheets of Whatman 3 MM paper and chromatographed 24 hr in solvent 1 and 4 hr in solvent 2.

We have similarly chromatographed flowers of all the species concerned, and although flower extracts offer much promise for future investigations, there were actually too many substances to provide a practical illustration of the techniques. We know that additional species-specific components are present in flowers. In *B. laevicaulis*, different chromatographic patterns are characteristic of the rhizome, stem, fruit wall, and seeds, and it is likely that all of these organs will contribute some useful compounds for future populational analysis.

Each chromatogram was examined in daylight in ammonia vapor, in ultraviolet light, and in ultraviolet light in the presence of ammonia and was finally sprayed with diazotized *p*-dinitroaniline, a general phenol-detecting reagent. Each visible component was checked out in all detecting procedures and assigned an identifying number. Four categories of spots warrant brief notation: (1) substances apparently common to two or even all three species, (2) substances which are species-specific and highly reliable because of distinctive features and consistent presence, (3) species-specific substances which are not constant for the species (these are often, though not



FIGS. 1–6.—Figures 1–3 represent composite two-dimensional chromatograms of leaf extracts of *Baptisia leucantha* (upper left), *B. viridis* (upper right), and *B. laevicaulis* (middle left). Figures 4–6 represent individual hybrids: *B. laevicaulis* × *B. leucantha*(middle right); *B. laevicaulis* × *B. viridis* (lower left); *B. leucantha* × *B. viridis* (lower right).

Black spots represent compounds of doubtful value in the cases indicated (perhaps characteristic of both parental species of a particular hybrid); dotted spots represent species-specific but minor components which are useful when present; grey (or finely stippled) spots represent major spots of greatest significance. La = *B. laevicaulis* spots; Le = *B. leucantha* spots; V = *B. viridis* spots and H = hybrid-specific spots.

always, present in low concentration and in some cases may simply be below the detecting threshold), and (4) hybrid-specific substances (a few of these are consistently present in hybrids, but not in the parental types). They are especially notable in hybrids involving *B. leucantha*. They may be *de novo* products of hybrid gene combinations or accumulations of substances normally produced only in small amounts in one parent or the other. The latter phenomenon has been described by other investigators and has been attributed to a breakdown of normal homeostatic mechanisms, thereby allowing accumulation of some useless components.[4] Whatever may be the explanation for these distinctive hybrid substances, described elsewhere,[1] they are quite interesting and of considerable theoretical importance.

TABLE 1

PERCENTAGE REPRESENTATION OF EACH OF THREE SPECIES IN INDIVIDUAL PLANTS OF TRI-HYBRID POPULATION AS INDICATED FROM MORPHOLOGICAL HYBRID INDEX

Plant No.	% *vir.*	%*lae.*	% *leu.*	Plant No.	% *vir.*	% *lae.*	% *leu.*
1	62	31	7	27	35	46	19
2	37	50	13	28	43	55	2
3	100	—	—	29	18	68	14
4	31	47	22	30	—	—	100
5	40	39	21	31	39	36	25
6	—	100	—	32	34	50	16
7	10	81	9	33	50	18	32
8	90	9	1	34	37	40	23
9	42	36	22	35	48	37	15
10	35	—	65	36	24	50	26
11	54	—	46	37	43	32	25
12	51	39	10	38	33	42	25
13	—	—	100	39	27	46	27
14	39	4	57	40	23	37	41
15	33	40	27	41	22	37	41
16	32	42	26	42	25	52	23
17	100	—	—	45	69	6	25
18	50	33	17	46	43	5	52
19	88	1	11	47	36	6	58
20	37	38	25	48	—	—	100
21	5	86	9	50	—	80	20
22	25	55	20	51	—	80	20
23	38	52	10	52	—	100	—
25	12	77	11	53	98	1	1
26	49	29	22				

Results and Interpretation.—The morphological hybrid index suggested that complex hybridization involving all three species in interaction occurred. Note especially plants numbers 36, 39, and 42 (Table 1), which appear to be *B. viridis* × *B. leucantha* hybrids crossed to *B. laevicaulis*. The chromatographic data, in contrast, indicated that *B. leucantha* × *B. viridis* and *B. laevicaulis* × *B. viridis* hybrids occurred, but no mixing of *B. leucantha* and *B. laevicaulis* genomes was detected. Although evidence on this point is not unequivocal, chromatographic data do not suggest any complex backcrossing, but rather it appears that most of the hybrid types are F_1. In the writers' opinion, the chromatographic evidence is so compelling that we conclude that the morphological hybrid index is providing some spurious evidence, particularly in the extent of complex tri-hybrid crosses implied by the data. That implications of the chromatographic data so sharply contrast with those derived from the morphological evidence in this case is indicative that chromatographic studies represent a potentially important advance in analyses of natural hybridization.

The *Baptisia* species contain a surprisingly large number of species-specific compounds, doubtlessly many more than we can presently distinguish. *B. leucantha* and *B. viridis* differ by at least 20 reliable constituents. When such a large pool of species-specific components is available, it is possible to carry an analysis of hybridization beyond a simple documentation of hybridization. For example, the extent and direction of backcrossing may be discovered if the following theoretical considerations hold. The hybrid A × B should have approximately the sum of the constituents of the parents, and a backcross to species A should have the compounds of A and approximately half the species-specific compounds of B. Since, in a real situation, we do not know the chemical composition of A × B or its limits, or what the practical limits of the chemical make-up of backcross types may be, these can

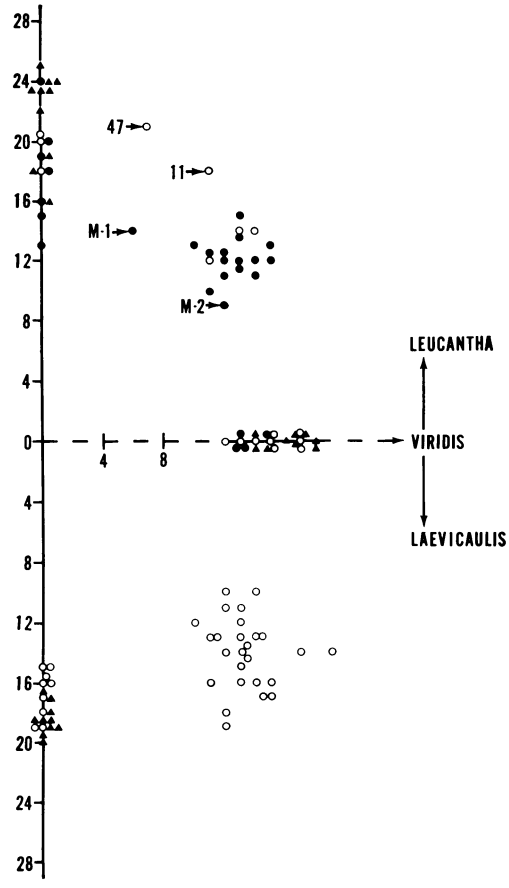


FIG. 7.—A three-way plot of individual hybrid types and pure species. Open circles indicate plants from tri-hybrid population, closed triangles indicate miscellaneous supplementary plants from pure populations, and closed circles indicate the additional (supplementary) *B. leucantha* × *B. viridis* hybrids. Points along the *X*-axis represent the number of compounds recognized of *B. viridis;* points along the *Y*-axis represent (above) the number of compounds recognized of *B. leucantha* and (below) the number of compounds recognized of *B. laevicaulis*. Hybrids fall at some angle between the *X* and *Y* axes.

be determined only after the analysis of a large population or many populations of hybrids. To illustrate, all three pure species and hybrids between *B. viridis* and *B. leucantha* have been plotted in Figure 7. All individuals from the tri-hybrid population are included, plus 10 individuals from pure populations of each species and 16 additional *B. viridis* × *B. leucantha* hybrids. Points in Figure 7 for hybrid types are calculated using both species-specific components and those shared in common by the *two* species considered involved, on the basis of the detectable components. In the case of *B. viridis* × *B. leucantha* hybrids, the plotting of spots shared in common does not greatly interfere with the interpretation of the table, since only a few compounds are shared. In the case of *B. viridis* × *B. laevicaulis* hybrids, more compounds are shared and fewer species-specific compounds are available for analysis of possible backcrossing. Therefore, we do not consider that those data representing the *B. viridis* × *B. laevicaulis* hybrids may be applied with validity to

the question of possible backcrossing. The plot of the data suggests little or no backcrossing, but we regard the question as open. Accordingly, we must concentrate on accumulating additional species-specific components of these two species.

In *B. viridis* × *B. leucantha* hybrids, where 80 per cent or more of the spots are representative of species-specific substances, it is evident that the hybrid-types cluster at a point at approximately a 45° angle above the *B. viridis* axis, indicating approximately equal representation of *B. leucantha* and *B. viridis* components. The hybrid-type plants have approximately a full complement from each parent species. The slight reduction stems from a general decrease we have observed in the quantities of these substances noted in hybrids, and thus a few minor components are missed. Differences in the plotted position of nonhybrid plants (Fig. 7) involve, principally, similar quantitative variations reflecting intrinsic differences, either genetic or environmental in cause, or perhaps inconsistencies of technique. Although we do not yet know the possible range of environmental effects, it is evident that the basic pattern of major components of each pure species is predictable.

Two plants from the tri-hybrid group (47 and 11) and one other (M-1) fall sufficiently outside the area of greatest concentration to suggest that these three plants may be hybrids backcrossed to *B. leucantha*. The morphological hybrid index suggests that they are F_1 hybrids.

In order to get some further insight into the question concerning the status of the *B. leucantha* × *B. viridis* hybrid group, ten major species-specific components from each of the two species were selected and their distribution among the hybrid-type plants noted (Table 2). Using this comparison as a basis, tri-hybrid #47 and also M-1 are suggested as possible backcross types to *B. leucantha*, and M-2 is suggested as a possible backcross type to *B. viridis* although its plotted position in Figure 7 is not out of line. Tri-hybrid #11 is more intermediate in position in Table 2.

TABLE 2

THE NUMBER OF MAJOR SPECIES-SPECIFIC SUBSTANCES OF EITHER *B. leucantha* OR *B. viridis* OF THE HYBRIDS SHOWN IN FIGURE 7

Tri-Hybrid Population		Miscellaneous Supplementary Hybrids	
B. leu.	*B. vir.*	*B. leu.*	*B. vir.*
8	7	8	10
8	7	10	8
8	7	9	9
8	6 (#11)*	8	8
9	6 (#47)*	7	8
		8	9
		7	7
		7	7
		8	9
		9	9
		7	9
		7	9
		7	8
		9	7
		8	4 (M-1)*
		5	10 (M-2)*

* See text for discussion of these plants which are also designated in Figure 7.

It is not the purpose of this report to settle the status of these individual plants. In the first place, considerable refinement and extension of the chromatographic data are possible. Secondly, an analysis of a much larger group of hybrids of *B. viridis*

and *B. leucantha* is required, in our opinion, before an interpretable pattern will emerge. It is highly probable that we can double the number of *B. viridis* or *B. leucantha* species-specific components and, subsequently, analyze from 500 to 1,000 hybrid-type plants. When these data are plotted, the pattern should suggest the amount of backcrossing occurring and even the presence or absence of favored backcrossing, for secondary concentrations of points at positions along angles of $22^1/_2°$ and $67^1/_2°$ from the *B. viridis* axis would imply the presence of backcross types. Because of segregation in the F_1, these concentrations may be less compact than that representing the F_1 group. If, by chance, the hybrids tend to be self-fertile, there would be expected no significant secondary concentrations and a somewhat diffuse primary concentration. We believe that this type of population analysis is not only quite practical in the case of *Baptisia* hybrids but in addition represents a significant advance over the use of morphological criteria alone. Although *Baptisia* seedlings do not flower until the second or third year, a crossing program to be initiated this year should yield information about biochemical compositions of the F_1 individuals from leaves in the first year.

Two plants, apparently hybrids of *B. leucantha* × *B. viridis*, on the basis of their exomorphic features, contained several minor components in the usual amounts but contained only small amounts of the major components, and a number of major components were missing. These two plants were twice rechromatographed (from new extractions), and all three of the results were similar. The plants are not plotted in Figure 7. As a working hypothesis only, it may be assumed that these plants are selfed F_1 hybrids. If multiple gene systems effect certain classes of species-specific compounds formed in a step-wise synthesis, then segregation patterns in selfed hybrids might interrupt the synthesis, thus accounting for a reduction in the number of compounds formed. If multiple gene systems effected the synthesis of major species-specific components indirectly, through favoring the accumulation of the compounds, similar reductions in these compounds would be expected in F_2 segregants. This point is of considerable importance because if this hypothetical explanation is correct, then an F_1 plant backcrossed to species A would have greater than expected reduction of B constituents, and some backcrossed individuals might be missed.

The rather wide range of morphological variation in the plants of the tri-hybrid population is puzzling and so, to a lesser extent, is the variation in the chromatographic patterns. As noted, the species are normally prone to cross-pollination and there must be, correspondingly, a greater degree of heterozygosity. More importantly, as mentioned before, the biochemical data do not suggest the absence of backcrossing of *B. viridis* × *B. laevicaulis* hybrids. These hybrids were more numerous in the tri-hybrid population as indicated from the data of Figure 7. Some of the apparent disparity between the morphological and biochemical data may actually involve failure to detect backcrossing involving the two last-named species and their hybrids.

The chromatographic data are not construed to disprove the existence of gene flow between the three species considered, even in the specific tri-hybrid population studied. They do, however, suggest a more conservative appraisal of the extent and nature of patterns of hybridization than might be inferred from the morphological data alone. In fact, we regard the chromatographic data as indicating that

the majority of all hybrids detected are F_1 hybrids, and that gene exchange between *B. laevicaulis* and *B. leucantha*, directly or indirectly, is rare in the tri-hybrid population studied. The existence of one definite hybrid between these two species, not from the tri-hybrid population, indicates that no absolute barriers to gene exchange between all three species occur.

The roughly 50 hybrid-type plants collected from the tri-hybrid population represented most of the readily recognized hybrid-types and constitute not more than about 5 per cent of the population. The life-expectancy of these perennial plants may be conservatively estimated at 20 years or longer. If the population density stays roughly constant, then each year only about 5 per cent of the population is replaced. Assuming no decreased fertility in the F_1 hybrids and the unlikely fact that the F_1 hybrids contribute in due proportion to the populational turnover, then only 0.25 per cent of backcross types per year could appear at a maximum. Under these conditions eventually a highly complicated (genetically) population would arise. The morphological and biochemical data differ in their implications concerning the present status of the population. It will be interesting to discover where in between the truth lies. We believe that further refinements of the biochemical methods coupled with genetic analyses will provide the answer.

Summary.—In the legume genus *Baptisia*, in which natural hybridization between quite distinctive species is common, three or even four species may be sympatric in a particular population. In cases of such actual or potential complex hybridization, a morphological hybrid index cannot adequately describe the situation. In a large tri-hybrid group of *Baptisia laevicaulis*, *B. leucantha*, and *B. viridis*, 50 hybrid type plants were analyzed by means of a morphological hybrid index utilizing 20 characters. Results indicated complex three-way hybridization with gene-flow between all species. The individuals were analyzed by two-dimensional chromatography of leaf extracts after preliminary work on the pure species had disclosed the presence of numerous species-specific components among the three species. Results of the chromatographic analyses were directly contrary to the implications of the morphological hybrid index. Numerous hybrids of *B. laevicaulis* × *B. viridis* and of *B. leucantha* × *B. viridis* were identified unequivocally by chromatographic evidence, but no *B. laevicaulis* × *B. leucantha* hybrid was found in the tri-hybrid population, and backcrossing of a hybrid between any two species to the third was not evident. The writers consider that the chromatographic data provide a more accurate indication of the population structure of the tri-hybrid group. Applications of the chromatographic techniques to other questions relevant to the population dynamics of natural hybridization were described.

* Supported by a grant from the National Science Foundation G-15890.

[1] Turner, B. L., and R. E. Alston (in preparation, 1962).

[2] Turner, B. L., and R. E. Alston, *Amer. J. Bot.*, **46**, 678-686 (1959).

[3] Larisey, M. M., *Amer. Jour. Bot.*, **27**, 624–628 (1940).

[4] Schwarze, P., *Planta*, **54**, 152–161 (1959).