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The Origin of *Lamium hybridum,* A Case Study in the Search for the Parents of Hybrid Species

Abstract

An unusual ("new") form of *Lamium* was observed growing sympatrically with *Lamium amplexicaule* L. and *L. purpureum* L. in northwestern Washington. The new taxon was noted to have characteristics more or less intermediate between these species, suggesting hybrid derivation. An observed lack of morphological segregation also suggested allopolyploidy. Taximetric (based on morphological and flavonoid variables), electrophoretic, and cytological analyses were carried out to test the hypotheses that the new taxon is a hybrid between *L. amplexicaule* and *L. purpureum*, that it is an amphidiploid (allopolyploid), and that hybridization occurred *in situ*. The experimental results confirm that the intermediate is a tetraploid and strongly implicate *L. purpureum* (2*n*) as a parental species. However, the results equally imply that *L. amplexicaule* (2*n*) is not the second parent; obviously, the new (hybrid?) species was not produced *in situ* but was introduced. A review of the literature and search of selected herbaria resulted in the identification of the new taxon as *Lamium hybridum* Vill., a European species. So, while the results of this study do not support the hypothesis of a new case of allopolyploidy, they exemplify the methodology used in tracing the parentage of hybrid species.

Introduction

I recall as a graduate student, while very much in the formative years of my profession, being told by my mentor, Marion Ownbey, that one of the primary responsibilities of systematists was to observe and document evolution at work. Certainly, Ownbey met that responsibility with his discovery and documentation of *Tragopogon mirus* and *T. miscellus*, amphidiploids (allopolyploid species) derived from hybridization of *Tragopogon dubius* X *T. porrifolius* and *T. dubius* X *T. pratensis* respectively (Ownbey 1955). Marion Ownbey was a first class plant systematist and a meticulous researcher; but he was also extremely lucky. Although we accept the premise that more than half of all angiosperm species were derived from hybridization followed by chromosome doubling (allopolyploidy), his are two of the few cases where this has actually been observed to occur naturally. So it was that in the spring of 1986 I became very excited upon discovering a small population of what appeared to be a new hybrid species. The new taxon was growing sympatrically with *Lamium amplexicaule* L. and *L. purpureum* L.; it was more or less intermediate between these two species; and, very importantly, there was very little variation among the plants, suggesting a stabilized F1 population rather than a hybrid swarm. Furthermore, the plants were more robust than the two known species, suggesting that they were polyploids-allopolyploids! Several questions came to mind: (1) Was this in fact a hybrid species? (2) Was it an allopolyploid? (3) If it were a hybrid, did hybridization occur *in situ* or was the plant introduced as a hybrid? (4) If the plant were an introduced hybrid, were the two parents *L. amplexicaule* and *L. purpureum*? (5) If the plant had been introduced, had it previously been described? The study that ensued was designed to answer these questions and typifies standard methods of biosystematic analyses.

Materials and Methods

Descriptive notations and experimental taxa

Lamium amplexicaule L. and *L. purpureum* L. are diploid species ($2n = 18$) originating in Europe but widely distributed in North America. Both are winter annuals and are common weeds in moist, disturbed habitats, including irrigated fields and orchards of the Northwest. The flowering stems of both are erect or slightly spreading from the base; the leaves are opposite and deeply toothed or lobed. The primary distinguishing characteristics (*L. amplexicaule* vs. *L. purpureum*) are: leaves wider than long, rounded at the tip, and the upper sessile and not crowded nor pigmented vs. leaves longer than wide, acute, and all petiolate, the upper crowded and reddish pigmented; corolla tube 9-11 mm long, thrice as long as the upper lip, and corolla bright lavender in color vs. corolla tube 4-6 mm long, twice as long as the upper lip, and corolla pale lavender. The third taxon, referred to as *L. "hybridum"* for convenience, has leaves resembling *L. purpureum* but somewhat larger, more deeply lobed, less crowded above and only slightly pigmented; the flowers are intermediate in size and shape but are colored similarly to those of *L. amplexicaule;* in habit, the plants are more robust, extensively branched and widely spreading. In the study areas (Figure 1), the three taxa occur sympatrically.

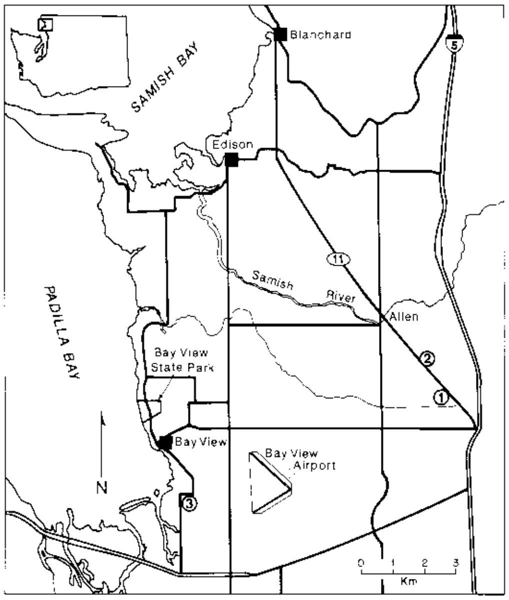

Figure 1. Map showing established populations of *Lamium hybridum* in Skagit County, Washington.

Field Sampling and Data Collection

In April, 1986, at the original site of observation (site 1, Figure 1), a transect was established through the most dense part of the population, paralleling highway 11. At five meter intervals along the transect, two 1-meter-square plots were located, one on each side of and one meter from the transect, for a total of 58 plots. Individuals of the three taxa were counted and frequency and abundance were calculated. Twenty suitably mature plants were collected along the transect, five of *L. purpureum*, four of *L. amplexicaule*, and eleven of *L. "hybridum."* These numbers were indicative of the relative abundance of the three taxa. In May of 1987 and again in 1989, additional collections were made from a dense sympatric population at site 2 (Figure 1). The purpose and nature of these collections are noted below under appropriate headings.

Morphological Measurements and Comparisons

A total of 24 morphological variables (listed in Table 1) was selected and measured on 60 specimens, 20 of each taxon. These specimens included the 20 plants collected from site 1 (and noted above), and 40 plants collected in 1989 along two 30 m transects at site 2. All plants selected for the analyses were in a similar stage of anthesis and measurements were made on fresh plants. The morphological relationships among the 60 specimens were determined using a principal components analysis (SPSSx 1984), the most common type of multivariate analysis in biosystematic studies.

Flavonoid Analyses

Thirty suitably mature plants, ten of each taxon, were randomly collected at site 2 in 1987. The flowers were removed and from these anthocyanins were extracted in 1% methanolic HCl. From the resulting extracts, anthocyanins were isolated, characterized, identified, and quantified using methods of paper chromatography as described by Taylor (1987), including spectral anslysis and hydrolysis. After the flowers had been removed, the plants were air dried, crushed, and flavonoid pigments were extracted in 80% methanol. Using standard methods of paper chromatography (Markham 1982) and spectral analysis (Mabry *et al.* 1979), the flavones and flavonols were isolated, characterized, quantified, and largely identified. Flavonoid (including anthocyanin) similarities (similarity values or SVs) among plants were determined by application of the formula: SV = similarities/similarities + dissimilarities (SV = s/s+d), first used by Sokal and Sneath (1963). The co-occurrence of a particular compound in two plants was given a similarity of three; an additional value of two was assigned if the compound was of the same scored concentration, one if the concentrations differed by a single scored value; Similarities + dissimilarities = 5. A pigment not occurring in either plant was not considered in determination of similarity. From these data, a covariance matrix (SPSSx 1984) was generated and used to construct a phenogram and dissimilarity triangle.

Flavonoids are the most widely used of all secondary metabolites in biosystematic studies. They are stable, can be analyzed from small

TABLE 1. Morphological variables used in comparative analyses.

1	– plant height (cm)
2	– mean leaf length (mm)
3	– mean leaf width (mm)
4	– length of stem (mid) leaf petiole (mm)
5	– upper leaves clasping = 1; non-clasping = 2
6	– depth of (mid) leaf margin incisions (mm)
7	– leaf venation pattern: interveinal space reduced and non-raised = 1; interveinal space med. and slightly raised = 2; interveinal space greater and more raised = 3
8	– leaf pubescence: sparse on both surfaces = 1; sparse on lower surface = 2; relatively dense on both surfaces = 3
9	– length of lowest internode (cm)
10	– calyx length (mm)
11	– calyx pubescence (character states: sparsely strigose = 1; moderately strigose = 2; sericeous = 3)
12	– calyx straight = 1; divaricate = 2
13	– corolla length (mm)
14	– corolla tube length (mm)
15	– galea length (mm)
16	– corolla color: pink = 1; lavender = 2
17	– color of galea pubescence: white = 1; white with pink base = 2; pink to purple = 3
18	– ring of hairs inside base of corolla tube: absent = 0; present = 1
19	– corolla pubescence (outside): absent = 0; sparse = 1; moderate = 2; heavy = 3
20	– filament pubescence: absent = 0; sparse = 1; moderate = 2; heavy = 3
21	– leaf blade orientation: parallel to stem = 1; perpendicular to stem = 2; varied = 3
22	– purple pigmentation of upper leaves: absent = 0; trace = 1; light = 2; moderate = 3; heavy = 4
23	– verticels of flowers per stem (number)
24	– branches per flowering stems (number)

samples, and can be tentatively identified by few procedures (Harborne and Turner 1984).

Cytological Observations

In 1986, seeds of *L. "hybridum"* were collected (by Joe Arnett) at site 1, germinated, and seedlings were grown to maturity in a greenhouse. Young anthers were excised from 20 immature flower buds and squashed in 2% aceto-orcein. From these buds, only one preparation showed meiotic figures clear enough to allow chromosomal counts. Several microsporocytes were examined and although precise counts were difficult, they consistently approximated $n = 18$ (Arnett, unpublished). As noted by Arnett and Bernstrom (1955) before him, suitable meiotic figures are difficult to obtain in *Lamium* because of very small chromosomes and few anthers. In June of 1988, seeds collected from plants of site 2 were germinated in petri dishes and root tip squashes were made of 10 seedlings each of *L. "hybridum"* and *L. amplexicaule*, and five of *L. purpureum*, the seeds of which were difficult to germinate. The squashes were stained with aceto-orcein and mitotic figures were examined. Finally, pollen samples of all plants used in morphological analyses were stained with acetocarmine, to get an estimation of viability, and measured.

Transplantations

Numerous specimens of all three taxa were transplanted from site 2 into an experimental garden at Western Washington University, Bellingham, WA. Also, seedlings of *L. "hybridum"* and *L. amplexicaule* were planted in the garden.

Isozyme Analysis

Ten flowering specimens of each taxon collected from site 2 in April of 1989 and ten specimens of *L. "hybridum"* from the experimental garden were sent to Washington State University for isozyme analysis. The analysis was done by Paul Wolf from the lab of Doug Soltis. Twelve enzymes or enzyme systems were surveyed electrophoretically.

Results and Interpretations

Population Structure

The percent frequencies and abundance (plants/sq. meter) of the three taxa at site 1 were: *L. amplexicaule* 17 and 0.12; *L. purpureum* 12 and 0.22; *L. "hybridum"* 76 and 2.29. Clearly, *L. "hybridum"* is more invasive or at least reproductively successful, an observation of potential consequence in dealing with northwest weeds. At site 2, although the population structure was not determined,

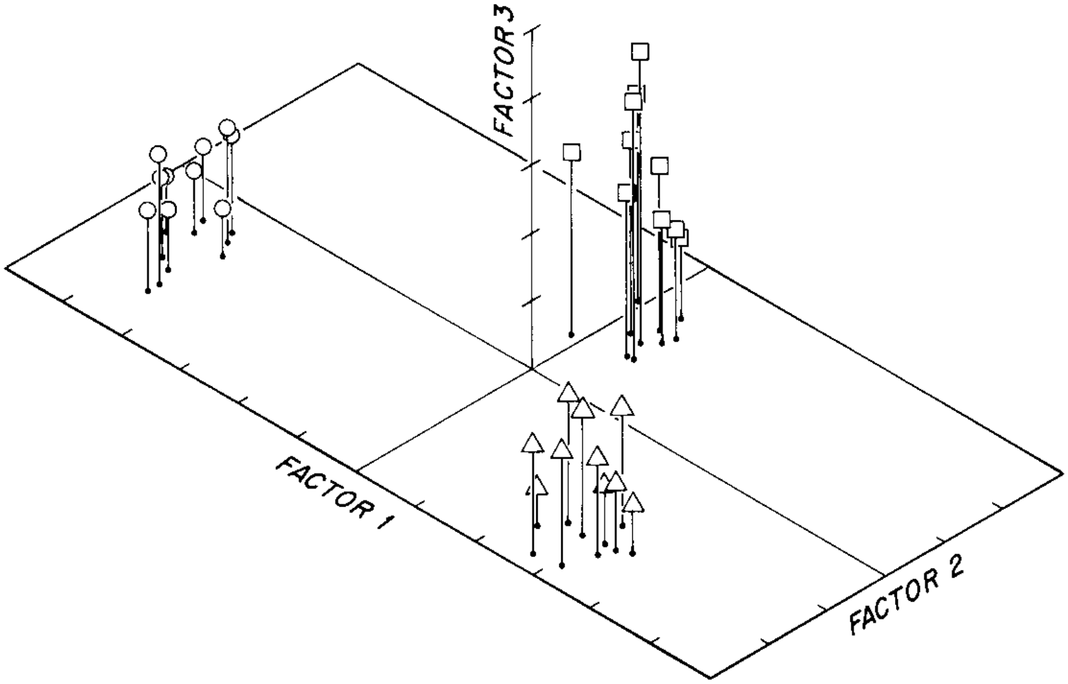

Figure 2. Ordination of odd-numbered plants based on principal components analysis; factors = components. Circle: *L. amplexicaule;* triangle: *L. purpureum;* square: *L. hybridum.*

L. "hybridum" appeared to be even more predominant.

Morphological Relationships

The principal components analysis shown in Figure 2 indicates the morphological relationships of the three taxa. Only the odd numbered specimens are plotted on the chart, including specimens from both collection sites. The analysis yielded six components with eigenvalues greater than one, indicating that the variables are moderately correlated (an inverse relationship exists between the number of components with high eigenvalues and the extent of correlation among variables). The first component (see Figure 2) accounted for 44.6% of the variance and separated *L. amplexicaule* from the other two taxa. The variables with the highest loadings, i.e., those that contributed most to the separation along component (factor) 1, listed according to loading values (high to low), were: upper leaves clasping vs. non-clasping (non-clasping in *L. amplexicaule* = *L.a.*); the presence or absence of a ring of hairs inside the corolla tube (absent in *L.a.*); pigmentation of upper leaves (non-pigmented in *L.a.*); calyx pubescence (sericeous in *L.a.*); corolla pubescence (heavy in *L.a.*); corolla tube length (longer in *L.a.*); and color of galea pubescence (pigmented throughout in *L.a.*). The second component accounted for 13.4% of the variance and separated *L. purpureum* from *L. "hybridum."* The variables with the highest loadings, from high to low, were: depth of leaf margin incisions (1/3 as deep in *L.p.*); leaf venation pattern (intervenal space less raised in *L.p.*); corolla pubescence (less dense in *L.p.*); branches per flowering stem (fewer or none in *L.p.*); and corolla length (shorter in *L.p.*). The third component accounted for 9.9% of the variance with only two variables having high loadings: leaf length and leaf width, both of which further separated *L. amplexicaule* (leaves longer than wide in *L. purpureum* and *L. "hybridum"* and vice versa in *L. amplexicaule*).

Perhaps the most significant result of the principal components analysis is the fact that the first component accounted for such a large proportion of the variance and that *L. amplexicaule* separated from the other taxa along this component. This suggests that the relationship between *L. purpureum* and *L. "hybridum"* is much greater than between *L. amplexicaule* and either or both of the other two taxa. This pattern of relationship is graphically portrayed in Figure 2. The results, then, of the principal components analysis do not support the hypothesis that *L. amplexicaule* is a parental species of *L. "hybridum"* but do suggest that *L. purpureum* is.

Comparative Flavonoid Chemistry

A total of 28 flavonoid compounds, 10 of which were anthocyanins, was isolated and used in analyses. The characteristics, distribution patterns, relative concentrations, and tentative identifications of the 18 flavones and flavonols are presented in Table 2. The identifications, occurrence and relative concentrations of anthocyanins are presented in Table 3. *Lamium "hybridum"* combines the anthocyanins of the other two species, suggesting their parental status. However, the occurrence of the other flavonoids does not support this interpretation; *L. "hybridum"* much more closely resembles *L. purpureum*, both in the sharing of flavones and flavonols and in their relative concentrations. In fact, all compounds found in *L. purpureum* also occurred in *L. "hybridum."* The most likely scenario, then, considering the results of the flavonoid analyses in conjunction with other data, is that *L. "hybridum"* shares flavones and flavonols with *L. purpureum* because the latter is a parental species; it shares anthocyanins with *L. amplexicaule* because the second and unknown parental species was similarly pigmented with malvidin glycosides. Also the observation that two compounds were found only in *L. amplexicaule* and two were unique to *L. "hybridum"* further supports this scenario. The presence of a greater number of flavonoids in *L. "hybridum"* supports the hypothesis of allopolyploid origin. The results of the chromatographic analyses are depicted in the phenogram and distance triangle of Figure 3. The co-occurrence of the malvidin glycosides in *L. amplexicaule* and *L. "hybridum"* skews the relationship in that direction.

Cytological Determinations

Although restricted to a single plant, chromosomal counts of microsporocytes of *L. "hybridum"* showed that taxon to be tetraploid ($n = 18$). Precise chromosomal counts of root tip cells of germinating seedlings were difficult to make, but the results were unambiguous in terms of ploidy level. In *L. "hybridum,"* 25 mitotic cells from seven seedlings were examined with a mean count of 34 ± 6 chromosomes. In *L. amplexicaule*, 19 cells from nine plants were examined with a mean count of 17 ± 3 chromosomes. In *L. purpureum*, 13 cells from five plants were examined with a mean count of 18 ± 2 chromosomes. These results are consistent with the published chromosome numbers of $2n = 18$ for *L. amplexicaule* and *L. purpureum*, diploid species. Also, this confirms that *L. "hybridum"* is a tetraploid, $2n = 36$. Pollen viability, based on stainability, was the same for all three taxa: 91 ± 7%, 90 ± 9%, and 91 ± 10%. The high pollen fertility (stainability) suggests that *L. "hybridum"* is an allopolyploid. However, Soltis and Soltis (1989) and Ness *et al.* (1989) have shown that autopolyploids may exhibit genetic equilibrium, thus high pollen fertility. Pollen grain size was significantly larger ($p = .05$) in *L. "hybridum"* as would be expected in a tetraploid. Relative mean sizes (length X width), standardized to 1, were: *L.a.* = 1.00, *L.p.* = 1.01, *L.h.* = 1.32.

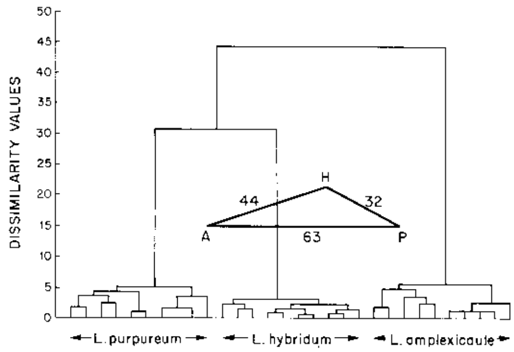

Figure 3. Phenogram and distance triangle showing relative similarities (dissimilarities) among experimental plants and species, based on flavonoid compounds. In the triangle, A = *L. amplexicaule*, H = *L. hybridum*, and P = *L. purpureum*.

Electrophoretic Analysis

From the twelve enzymes surveyed, 44 isozymes/allozymes were isolated; see Figure 4. Only two cases of heterozygosity within taxa were observed: one plant and one isozyme in each of the diploid species. The garden and field specimens of *L. "hybridum"* were homozygous for all isozymes. For convenience and because of this low percent of heterozygosity, the three taxa have been considered homozygous in Figure 4. Of the 44 isozymes, only four occurred in all three taxa; two were restricted

TABLE 2. Characteristics and Distribution Patterns of Flavonoids Compounds Isolated by Paper Chromatography.

Spot Number	Rf Values (BAW/HOAC)	Spot Appearance[1]		UV Spectrum[2]	Hydrolysate (Aglycone)	Tentative I.D.	Occurrence and Relative Concentration		
		UV	UV/NH_3	($AlCl_3$)			*amplex.*	*hybridum*	*purpureum*
1	40/53	PuAb	Y-OFl	257, 265s, 296s, 358 (430)	Quercetin	Q.3-rutinoside	+	++	++
2	42/13	PuAb	bYFl	255, 267s, 348 (423)	Luteolin	L.7-glucoside	++	++	++
3	57/27	PuAb	bYFl	254, 266s, 347 (424)	Luteolin	L-4',7-diglucoside	++	++	–
4	63/27	PuAb	Y-GFl	267, 336 (350, 383)	Apigenin	A-7-glucoside	++	++	+
5	58/45	PuAb	bY-Fl	255, 268s, 297, 356 (431)	Quercetin	Q.3 (acyl)-glucoside	–	++	++
6	37/53	PuAb	YFl	256, 266s, 297, 357 (430)	Quercetin	Q.3-diglucoside	++	+	+
7	82/06	PuAb	Y-OFl	253, 267s, 289, 348 (420)	Luteolin	Luteolin	+++	+	+
8	82/28	PuAb	Y-GFl	265, 297s, 314, 355s (395)	Kaempferol	K.3 (acyl)-glucoside	–	+	+
9	28/10	PuAb	Y-GFl	265, 295s, 318, 360 (400)	Kaempferol	K. glycoside	+	–	–
10	43/38	PuAb	Y-OFl	263, 342 (weak)	– – –	A. glycoside (?)	++	++	+
11	63/13	YFl	bYFl	– – –	– – –	K. 4'-glucoside (?)	+	++	+
12	63/17	PuAb	YFl	– – –	– – –	– – –	+	+	+
13	60/39	PuAb	bYFl	254, 265s, 295s, 356 (430)	– – –	Q.3-glucoside	+	++	++
14	58/59	PuAb	Y-GFl	266, 285, 336 (350, 395)	– – –	A.7 (acyl)-rutinoside (?)	–	++	+
15	58/46	PuAb	Y-GFl	265, 295s, 337 (345, 392)	– – –	A.7-rutinoside (?)	–	+	+
16	38/11	YFl	YFl	– – –	– – –	Q.7-glucoside (?)	+	+	+
17	40/71	PuAb	YFl	– – –	– – –	– – –	+	–	–
18	84/39	PuAb	YFl	– – –	– – –	– – –	–	+	–

[1]Abbreviations: Ab = absorption, Fl = fluorescence, G = green, O = orange, Pu = purple, Y = yellow, b = bright.

[2]Wave lengths (nanometers) in methanol; the shift with addition of $AlCl_3$ is indicated in parentheses.

TABLE 3. Anthocyanins—Occurrence and Relative Concentration

Anthocyanin[1]	*amplexicaule*	*hybridum*	*purpureum*
Cyanidin 3,5—diglucoside	++	++	++
Cyanidin acyl-3 glycoside	+	++	++
Delphinidin 3,5-diglucoside	+	++	++
Delphinidin 3-glucoside	−	+	+
Delphinidin acyl-glycoside	−	+	+
Malvidin 3,5-diglucoside	++	+++	−
Malvidin 3 rhamnose, 5-glucoside	+	++	−
Malvidin acyl-glycoside	+	++	−
Malvidin glycoside (1)	+	+	−
Malvidin glycoside (2)	−	+	−

[1]Identifications were based on Rf values with several developing solvents, spectral analyses, stepwise hydrolysis, and comparisons with known standards (aglycones).

to *L. amplexicaule* and *L. "hybridum,"* suggesting possible parentage by the former; fourteen were restricted to *L. purpureum* and *L. "hybridum"* and none was unique to *L. purpureum*, thus strongly implicating *L. purpureum* as a parent; fifteen were unique to *L. amplexicaule*, sufficient evidence to reject this taxon as a parent; and nine were unique to *L. "hybridum,"* suggesting an undetermined second parent. In summary, then, *in situ* hybridization between the diploid taxa can be ruled out on the basis of enzyme electrophoresis. Also it is unlikely that a different source of *L. amplexicaule* was the second parent, given its high level of enzymatic distinction.

Discussion

Interestingly, in 1786 Villars discovered a population of plants which he considered to be intermediate between *L. amplexicaule* and *L. purpureum*. Accordingly, he named his discovery *Lamium hybridum*. Subsequently, the question of the origin of the taxon has been widely debated. The early taxonomic history of *Lamium hybridum* Vill. was outlined by Little and Warburg (1953), beginning with an original description by John Ray. These authors cite several references voicing diverse opinions concerning hybrid origin, with *L. amplexicaule* and *L. purpureum* most frequently named as the likely parents. Little concludes that the "balance of evidence" is against hybrid origin. Warburg, being a much later contributor to the article, had the advantage of more recent cytological data (e.g., the tetraploid status) and agrees that hybridization probably was involved but emphasizes that *L. hybridum* and other related taxa "must be treated as independent species." According to Hegi *et al.* (1936), there are two naturally occurring forms of *L. hybridum:* ssp. *intermedium* and ssp. *dissectum*. These authors noted that both subspecies are intermediate between *L. purpureum* and *L. amplexicaule*, ssp. *intermedium* most definitely so. However, taxonomic revision (Tutin *et al.* 1972) placed ssp. *intermedium* in the separate species, *L. moluccellifolium* Fries, and ssp. *dissectum* became a synonym of *L. hybridum*. The description of *L. hybridum* Vill. fits that of *L. "hybridum"* and from this point the two will be considered synonymous.

Several earlier studies have attempted to establish the parental species of *L. hybridum* (Bernstrom 1941, 1944, 1949, 1955; Jones and Jones 1965; Jorgensen 1927; Little and Warburg 1953; and others). All consider *L. purpureum* to be a parent but *L. amplexicaule* is questionable, both because of a lesser morphological similarity and apparent reproductive isolation based on attempts to hybridize the two diploids. The most comprehensive hybridization studies were carried out by Bernstrom (1955), involving several *Lamium* species. He crossed *L. purpureum* with *L. bifidum* ($2n$) and obtained a sterile F1 hybrid which he treated with colchicine to derive a synthetic allopolyploid. This he crossed with *L. hybridum* and obtained a fertile tetraploid with normal bivalent formation. He also produced a colchicine-derived autopolyploid of *L. purpureum* which he also

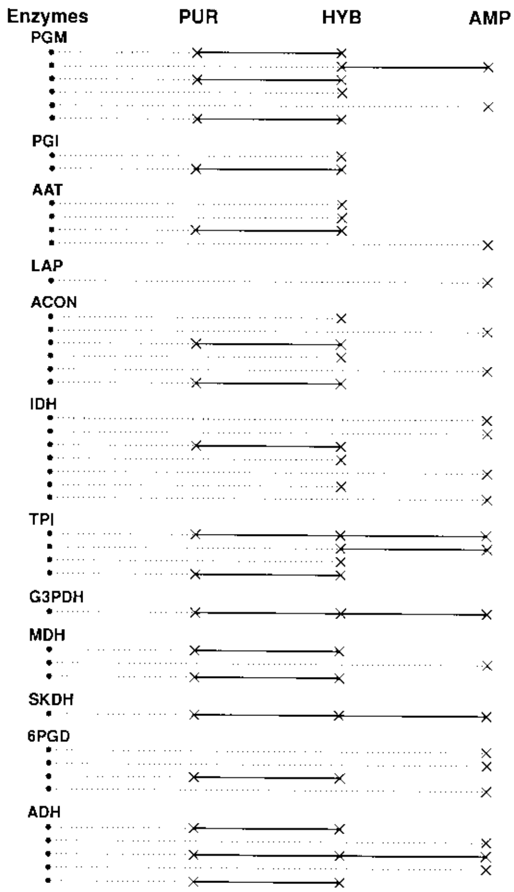

Figure 4. Distribution of 44 isozymes/allozymes as determined by enzyme electrophoresis. PGM = phosphoglucomutase. PGI = phosphoglucoisomerase, AAT = aspartate dehydrogenase, LAP = leucine aminopeptidase, ACON = aconitase, IDH = isocitrate dehydrogenase, TPI = triosephosphate isomerase, G3PDH = glyceraldehyde-3-phosphate dehydrogenase, MDH = malate dehydrogenase, SKDH = shikimate dehydrogenase, 6PGD = 6-phosphogluconate dehydrogenase, ADH = alcohol dehydrogenase.

crossed with *L. hybridum.* The result was a semifertile 4*n* hybrid which produced trivalents at meiosis, presumably due to three sets of chromosomes from *P. purpureum.* These results suggested that the parents of *L. hybridum* are *L. purpureum* and *L. bifidum.*

Conclusions

The questions asked in the introduction can be answered: (1) Is *L. "hybridum"* a hybrid species? The flavonoid, morphological, cytological, and electrophoretic data support this hypothesis, with *L. purpureum* being one of the diploid parents. (2) Is *L. "hybridum"* an allopolyploid? Certainly the cytological data in concert with the electrophoretic and other experimental results support this hypothesis. (3) Did hybridization occur *in situ*? The unescapable conclusion is that it did not. (4) If *L. "hybridum"* is an introduced hybrid, are the two parents *L. amplexicaule* and *L. purpureum*? The results of this study strongly suggest that *L. purpureum* is but *L. amplexicaule* is not. (5) Had *L. "hybridum"* previously been described? Yes, coincidently, it matches the description of *L. hybridum* Vill. and is similar to the few specimens of *L. hybridum* Vill. observed by the author at the U.S. National Herbarium (US), the University of Maryland (MARY), and the University of Massachusetts (MASS). A final question, then, would be: What species is the second diploid parent (assuming that only one additional species was involved, which perhaps is a bit presumptuous)? My data can not be addressed to this question, but the work of Bernstrom (1955) would suggest *L. bifidum.* In spite of my attempts, I have not yet been able to obtain seeds of this European species to test this hypothesis.

Acknowledgments

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