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Hybridization and Introgression in Buckeyes (*Aesculus*: Hippocastanaceae): A Review of the Evidence and a Hypothesis to Explain Long-Distance Gene Flow

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ABSTRACT. Evidence from morphology, distribution patterns, allozyme variation, and meiotic irregularities associated with decreased pollen germinability confirms the existence of a broad hybrid zone involving three parapatric species of *Aesculus* in the southeastern United States. The overall hybrid zone involving the three species is at least 200 km in width and probably represents the overlap of two hybrid zones: one between *A. pavia* and *A. sylvatica* and the other between *A. flava* and *A. sylvatica*. Both zones are highly asymmetrical, with hybrid populations occurring primarily in the Piedmont, where *A. sylvatica* is native. Detailed analyses of the hybrid zone involving *A. pavia* and *A. sylvatica* showed that hybrid populations consistently lack one or both of the putative parental species. Morphology and allozyme variation provide similar estimates of the position of the hybrid zone, but allozymes allow the detection of a larger zone than apparent on the basis of morphology. All available evidence is consistent with the hypothesis that extensive introgression has occurred among these species. Nevertheless, allozymic differentiation between these species is insufficient to reject hypotheses other than introgression that could generate the genetic structure observed in hybrid populations. Observations of pollinator activity in populations of *A. pavia*, *A. sylvatica*, *A. flava*, and their hybrids showed that these species share a number of important pollinators, including several species of bumblebees (*Bombus*) and the ruby-throated hummingbird (*Archilochus colubris*). Spring migration patterns of the ruby-throated hummingbird coincide closely with the flowering phenology of each of the *Aesculus* species, suggesting that hummingbirds could be vectors of long-distance pollen dispersal. The lifespan of pollen under field conditions is sufficient to permit transport of pollen in this fashion over distances of tens or hundreds of kilometers. Directional migration and arrival of hummingbirds after peak flowering would enforce a directional pattern of gene flow and could generate an asymmetrical hybrid zone of unusually great width.

Interspecific hybridization has long been considered a frequent and potentially important process in plants. Nevertheless, hybridization and introgression remain subjects of considerable controversy. Gottlieb (1972) and Heiser (1973), for example, emphasized the difficulty of documenting hybridization and introgression such that they may be distinguished from other situations that produce similar results. Heiser (1973) concluded that strong evidence for the importance of introgression as an evolutionary force was lacking because nearly all examples of introgression are highly localized in nature. Even when the case for hybridization is reasonably clear, Wagner (1969, 1970) argued that hybrids, in the absence of polyploidy, have limited evolutionary potential because their characteristics are "in between" those of two presumably well-adapted species. Levin (1979) pointed out that much remains unknown about hybridizing plant population systems, making it difficult to assess the real evolutionary impact of interspecific hybridization.

This lack of detailed understanding of hybridization in plants may be due, in part, to the fact that relatively few recent studies have examined hybrid zones in plants as a primary focus of investigation (Hewitt 1988; but see Bloom 1976; Flake et al. 1973; Heywood 1986; Levin 1975; Rieseberg et al. 1988; Soltis 1986; Wagner et al. 1987; Werth et al. 1985; Wheeler and Guries 1987). Documentation of hybridization has often been a by-product of investigations directed toward other goals, such as revisionary studies, in which detailed studies of hybrid population systems are usually, of necessity,

TABLE 1. Diagnostic morphological characters of the four species of *Aesculus* sect. *Pavia* and their putative hybrids. Data were compiled from Hardin (1957c, 1957d). Values from stamen length and calyx length are mean (range) in mm. Abbreviations are the first letter of the specific epithet. [a] Meyer and Hardin (1987).

Diagnostic character	Species or hybrid combination				
	pavia	*p* × *s*	*sylvatica*	*s* × *f*	*flava*[a]
Stamens (E = exserted; I = included; G = greater than upper petal)	E	E or I	I	I	I
Stamen length	31 (24–38)	25 (19–32)	20 (16–25)	19 (16–24)	13 (12–17)
Stipitate glands on pedicel	none	none	none	few, with trichomes	glandular trichomes
Petiolule length >3 mm or <3 mm	<	<	<	both	>
Petal margin (G = glandular; V = villous; P = pubescent)	G	G-V	V	V	V
Perianth color (Y = yellow; R = red)	R	Y-R	Y	Y	Y
Calyx length	15 (14–18)	12 (8–19)	10 (8–13)	9 (6–14)	7 (6–8) 10 (8–12)
Habit [Shrub or small tree (S); Large tree (T)]	S	S	S	S/T	T
Fruit spines and spacing	none	none	none	none	none
Lateral petal length (mm)	(20–31)	—	(20–30)	—	22 (19–24)

limited in extent. In contrast, and possibly *because* they are widely perceived as unusual (Mayr 1963) and, therefore, intrinsically interesting events (J. Avise, pers. comm.), hybrid zones in a variety of animal taxa have recently been the focus of extensive empirical and theoretical investigation (reviewed by Barton and Hewitt 1985; Hewitt 1988).

The contrast between perceived frequency of occurrence and lack of detailed understanding is particularly apparent in woody plants, where the list of reported hybrids is unusually large (Grant 1981; Stace 1975; Stebbins 1950). Grant (1981) suggested that this may be a by-product of the relatively great attention often directed by botanists to the woody plants of a region. Furthermore, reexaminations of reported cases of hybridization have shown that some "textbook examples" of hybridization and introgression in woody plants may actually be erroneous (Flake et al. 1973; von Rudloff 1975).

The purpose of this paper is: 1) to review evidence of hybridization and introgression in *Aesculus*, a particularly well-studied case of interspecific hybridization in woody plants, and present some additional evidence bearing on the hypothesis of hybridization among species of sect. *Pavia*, and 2) to examine features of the reproductive biology of these species in order to look for a possible mechanism of long-distance gene flow that could explain a number of unusual features of the hybrid zone between these species. We argue that extensive hybridization has occurred over a broad geographic area despite a largely parapatric distribution (Woodruff 1973) of the parental species. Furthermore, we propose that long-distance gene dispersal is effected by migrating ruby-throated hummingbirds (*Archilochus colubris*).

EXISTING EVIDENCE OF HYBRIDIZATION

Morphology. As part of a monographic revision of the genus *Aesculus*, Hardin (1955, 1956, 1957a, 1957b, 1957c, 1957d) examined more than 5000 herbarium specimens and living plants from much of their range in the eastern United States. Hardin (1957b) identified 10 diagnostic morphological characters, primarily reproductive traits, that he used to distinguish four species of *Aesculus* sect. *Pavia* (table 1). Each of these species was distinguished from all others throughout most of its range by 4–8 characters (table 1, fig. 1). In addition, a number of other consistent differences, including inflorescence structure (Hardin 1955), seed number and size

TABLE 1. Continued.

Species or hybrid combination				
f × (*s* × *p*)	*p* × *f*	*f* × *g*	*glabra*	*p* × *g*
E or I	E or I	E or I	E and G	E and G
20 (15–24)	—	—	(13–23)	—
few, with trichomes	glandular trichomes	few, with trichomes	none	none
both	—	—	—	—
G-V	G-P	V	V	G-V
Y-R	Y-R	Y	Y	Y-R
9 (6–11)	—	7 (4–11)	5 (4–6.5)	10 (6–12)
S/T	S/T	S/T	S/T	S/T
none	none	some, irregular	many, regular	some, irregular
—	—	15 (10–22)	10.5 (8–13)	24 (13–30)

(Schopmeyer 1974), fruit husk thickness, and the odor of broken stems, were also recognized but not included as "key" characters. Thus, throughout most of their respective ranges, these four species are morphologically and geographically distinct, a situation typical of the genus *Aesculus*, in which species are unusually well-marked (Hardin 1957b, 1957d).

Greatly complicating this situation, however, were plants from a large number of localities, primarily in northern Georgia, but also including parts of Alabama, Tennessee, and the Carolinas, which possessed various combinations of the diagnostic characters of *A. pavia* L., *A. sylvatica* Bartr., and *A. flava* Sol. (fig. 1). Other populations west and north of this region included plants that combined the diagnostic characters of *A. glabra* Willd. and *A. pavia*; other localities had plants that combined the traits of *A. glabra* and *A. flava*. Hardin (1957d) examined this situation using population samples consisting of 20–50 randomly selected plants from over 30 separate localities that were widely distributed throughout the range of the four species and the region of confusion. He used pictorialized scatter diagrams (Anderson 1949, 1953) to summarize the patterns of variation in the four species and populations from the zone of confusion. These showed that many plants had combinations of characters not expected for any of the species (Hardin 1957d). Further analysis showed that characters of the putative parental taxa were "loosely associated" (i.e., weakly correlated) in the suspected hybrid populations (Anderson 1953). By all of Anderson's (1953) criteria, the plants appeared to be hybrids, and a large number of plants with only one or two "alien" characters suggested much localized and dispersed introgression (Heiser 1973) over a broad area.

To summarize the patterns of morphological variation documented by Hardin (1957d), we performed a principal components analysis using data from populations of *A. pavia*, *A sylvatica*, *A. flava*, and putative hybrid populations (details in Methods, below). A plot of the first three principal component scores shows that each of the three species is well separated from the others and has relatively little overall morphological variation, as indicated by small average coefficients of variation (fig. 2). Putative hybrid populations occur in positions intermediate to their putative parents and are much more variable.

Cytology and Biochemistry. Additional evidence that these patterns of variation could

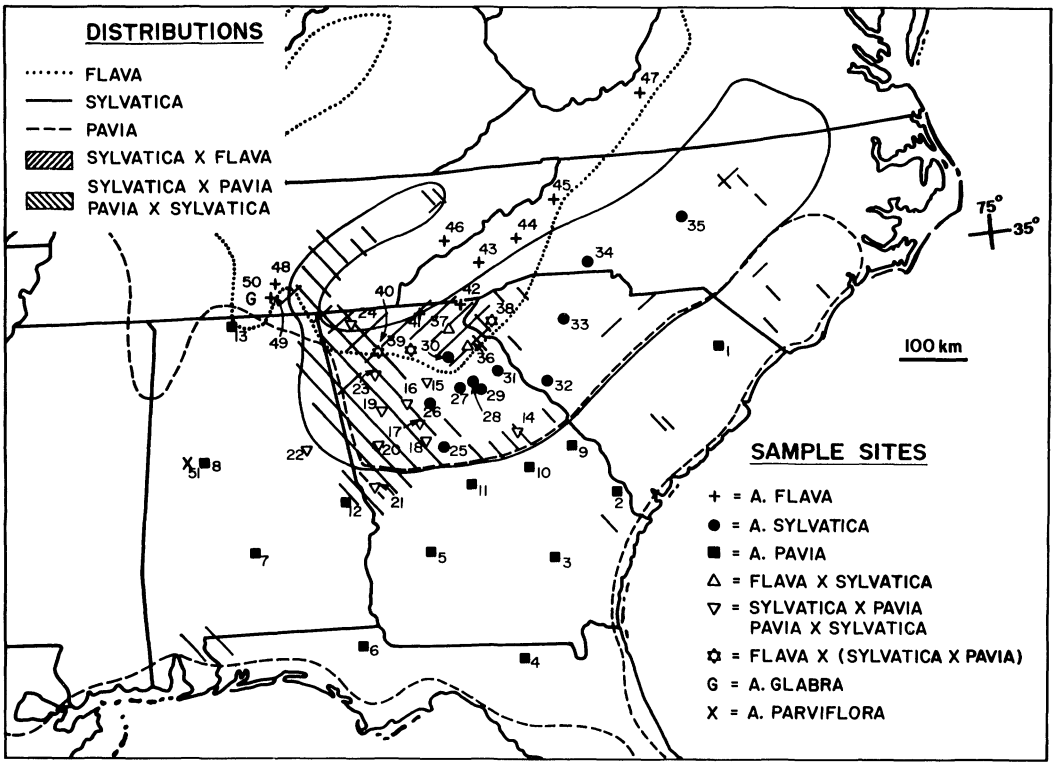

FIG. 1. Distribution of *Aesculus* species and hybrids (based on Hardin 1957d) in the southeastern United States and locations of sample sites used in the electrophoretic survey (dePamphilis 1988a; dePamphilis and Wyatt 1989).

be due to an extensive zone of hybridization included the observation that hybrids were produced commonly in gardens where two or more of the species were cultivated in close proximity (Hardin 1957c, 1957d). In addition, putative hybrid individuals frequently had various meiotic irregularities, including polyspory (Hoar 1927), lagging chromosomes (Hoar 1927; Pelletier 1935), and inviable pollen (Hardin 1957d; Hoar 1927). The occurrence of meiotic irregularities in suspected hybrids is a critical source of evidence to distinguish hybrids from members of an ancestral gene pool (Gottlieb 1972; Heiser 1973). Unfortunately, all such evidence in *Aesculus* is qualitative, in that no data were presented on the frequency of such irregularities in putative hybrid versus non-hybrid individuals. Cytological studies of *Aesculus* have mainly documented that all species (except *A.* ×*carnea* Zeyher, described below) share a uniform number ($n = 20$) of very small (ca. 1 μm) chromosomes (Hardin 1957d; Hoar 1927; Mehra et al. 1972; Skovsted 1929; Upcott 1936).

Flavonoid chemistry has been used successfully to document the hybrid origin of *A.* ×*carnea*, a fertile allopolyploid ($n = 40$) hybrid between *A. pavia* and the distantly related European horsechestnut, *A. hippocastanum* L. (Hardin 1957d; Hsiao and Li 1973). Additional biochemical evidence supporting the intersectional hybrid origin of *A.* ×*carnea* has been reported by Fowden et al. (1970), who analyzed the distribution of phenylalanyl-tRNA synthetases and non-protein amino acids in seeds of these species. Early reports of major differences in chromosome size between *A. pavia* and *A. hippocastanum* that could be used to document hybridization (Hoar 1927; Skovsted 1929) were challenged as artifactual (Upcott 1936).

Among the species of sect. *Pavia*, flavonoid chemistry appears to be of little use for the documentation of interspecific hybridization be-

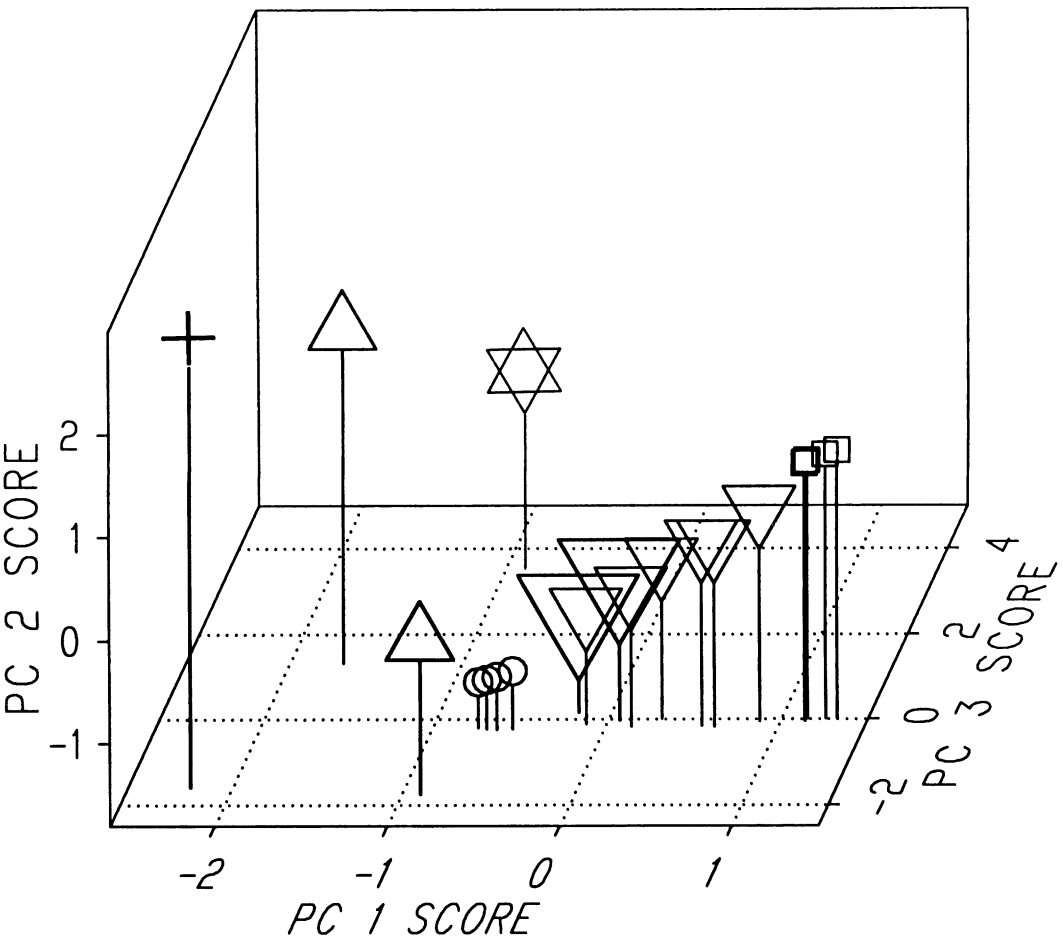

FIG. 2. Plot of first three principal components of morphological variation for 22 populations of *A. pavia* (□), *A. sylvatica* (○), *A. flava* (+) and their putative hybrids, using data from Hardin (1957d). Symbols for hybrid populations are: *A. sylvatica* × *pavia* (▽), *A. sylvatica* × *flava* (△), and *A. flava* × (*sylvatica* × *pavia*) (✡). The first three principal components account for 71.1%, 20.4% and 4.6% of the overall variation, respectively. Size of the symbol reflects the average coefficient of variation for all characters within a population.

cause these species share similar foliar flavonoid profiles (dePamphilis 1988b). Other biochemical similarities, including non-protein amino acids (Fowden et al. 1970) and carbohydrate chemistry (Pollard and Amuti 1981) suggest that these species have diverged little from each other in their basic metabolic pathways.

Allozymes. Recent studies of allozyme variation in these species of *Aesculus* and putative hybrid populations (dePamphilis 1988a; dePamphilis and Wyatt 1989) have supported and significantly strengthened most of Hardin's (1957b, 1957c, 1957d) conclusions. Over 1600 individuals were selected at random and sampled from within 48 populations of *A. pavia*, *A. sylvatica*, *A. flava*, and their putative hybrids, including nine of the populations previously studied by Hardin (1957c, 1957d). One additional population each of *A. glabra*, *A. parviflora* Walt., and a putative *A. glabra* × *flava* population were also sampled. Populations were classified as parental or hybrid based on the occurrence of the diagnostic morphological characters mentioned previously (Hardin 1957d; table 1). Nine enzyme systems encoded by 14 putative

TABLE 2. Frequencies of partially diagnostic alleles and gene admixture proportions (Elston 1971) for representative populations of four species of *Aesculus* and their hybrids (dePamphilis 1988a; dePamphilis and Wyatt 1989). Presumed source of alleles is indicated by first letter of specific epithet. [a] Mean of eight populations examined by dePamphilis (1988a). [b] Mean of seven populations examined by dePamphilis (1988a). [c] Single population examined by dePamphilis (1988a).

	A. pavia			*A. sylvatica* × *pavia*					*A. sylvatica*			*s* × *f*	*f* × (*s* × *p*)	*A. flava*		
Allele	Mean[a]	2	3	16	18	20	21	24	28	34	Mean[b]	36	40	41	Mean[b]	*A. glabra*[c]
Aat2-b	0.02			0.02	0.01	0.02						0.03			0.02	0.26
Aat3-a	0.07	0.08	0.05			0.02-SP	0.09-P	0.21-P								
-b	0.27	0.38	0.40	0.05	0.04	0.06	0.20-P	0.09-P	0.03		0.02	0.06	0.05			
-c				0.16-S	0.10-S	0.02			0.03	0.10	0.07					
-e	0.13	0.08	0.08	0.50-S	0.72-S	0.59-S	0.50-S	0.38-S	0.95	0.90	0.84	0.80	0.75	1.00	0.98	0.89
-h	0.52	0.46	0.40	0.28-P	0.12	0.32-P	0.19-P	0.32-P			0.07	0.06	0.20-P		0.02	0.11
Acp1-b	0.02		0.01	0.45-S	0.46-S	0.50-S	0.30-S	0.04	0.58	0.47	0.49	0.40	0.58	0.06	0.08	
Lap1-b	0.38	0.41	0.46	0.06	0.07	0.11	0.39	0.19	0.06	0.12	0.10	0.27	0.33	0.25	0.14	1.00
-c	0.58	0.59	0.53	0.24	0.22	0.48	0.40	0.40	0.50	0.36	0.47	0.38	0.33	0.33	0.55	
-d	0.04		0.01	0.42-S	0.34-S	0.22-S	0.06	0.25-S	0.19	0.37	0.24	0.18	0.02	0.43	0.18	
-e	0.01			0.29-S	0.36-S	0.19-S	0.14-S	0.16-S	0.23	0.15	0.18	0.17	0.29-FS		0.13	
Me1-b	0.01	0.04		0.03	0.13-S	0.02	0.11		0.05	0.36	0.25	0.06	0.08	0.19	0.07	0.50
Me2-c	0.88	0.95	1.00	0.57-P	0.11	0.98-P	0.80-P		0.22	0.13	0.16	0.69		0.37	0.27	
-d	0.12	0.05		0.18	0.89-S	0.02	0.20	1.00-S	0.78	0.87	0.84	0.32	1.00	0.64	0.73	1.00
Px2-e	0.72	0.60	0.70	0.57	0.76	0.62	0.55	0.78	0.81	0.70	0.64	0.57-S	0.22-SP		0.01	0.14
-g	0.01	0.01	0.01	0.05	0.03	0.19-S			0.14		0.17	0.14	0.59-F	0.90	0.87	0.77
-i	0.01	0.03	0.03				0.03					0.04-F	0.15-F	0.10	0.08	
Pgi1-d					0.03								0.02			0.23
Pgi2-a	0.14	0.05	0.09	0.16	0.08	0.13	0.17	0.18	0.24	0.08	0.18	0.38	0.22	0.52	0.46	
-d	0.21	0.05	0.18	0.04	0.01	0.09-P	0.17	0.49-P			0.01	0.03			0.02	
Pgm2-b	0.01	0.04		0.47-S	0.17-S	0.20-S	0.34-S	0.05-S	0.57	0.46	0.55	0.78	0.44-FS	1.00	0.90	0.52
-c			0.01	0.01	0.01	0.06-S		0.02-S	0.03	0.01	0.05		0.02-S			
-e	0.96	0.91	0.95	0.35	0.81	0.72	0.63	0.92	0.40	0.53	0.38	0.22-S	0.52-PS		0.04	0.46
Pgm3-d	0.09	0.10	0.18	0.06	0.11	0.41	0.34		0.12	0.26	0.28	0.22	0.10	0.50	0.48	0.60
Shk2-c	0.04	0.03	0.08	0.02-P				0.08-P				0.02-PF		0.08	0.05	
-e	0.05	0.14	0.01	0.76-S	0.86-S	0.74-S	0.51-S	0.09	0.95	0.73	0.90	0.99	0.65-SF	0.88	0.91	0.98
-g	0.87	0.78	0.90	0.13-P	0.08	0.07	0.40-P	0.83-P	0.04		0.02		0.35-P			0.02
	Admixture proportions for above populations															
A. pavia		0.96	1.00	0.31	0.17	0.46	0.56	0.65	0.01	0.02		0.16	0.14	0.00		
A. sylvatica		0.04	0.00	0.68	0.83	0.48	0.32	0.35	0.99	0.98		0.55	0.62	0.00		
A. flava		0.00	0.00	0.01	0.00	0.06	0.12	0.00	0.00	0.00		0.29	0.25	1.00		

TABLE 3. Summary of allelic character index scores (dePamphilis and Wyatt 1989) for populations of *A. pavia*, *A. sylvatica*, and hybrids, pooled across populations with similar composition. *P* is number of populations and *N* is number of individuals. Probable identifications of plants with certain character index scores are based on computer simulations described by dePamphilis and Wyatt (1989).

			Character index scores and probable identifications										
			A. pavia			Hybrids				*A. sylvatica*			Mean character index
Composition of population	*P*	*N*	(−)8–6	5–4	3–1	0–1	2–3	4–6	7–8	9–10	11–12	13–14(+)	
A. pavia	7	201	120	53	27	1							−5.24
A. pavia + hybrids	9	221	41	64	72	24	11	8	1				−2.81
Hybrids	3	65		1	17	16	19	10	2				+1.17
A. sylvatica + hybrids	7	164			2	9	9	61	42	29	12		+6.46
A. sylvatica	9	184					1	30	62	74	15	2	+8.29

gene loci were resolved and used for all analyses. Progeny analyses of open-pollinated seed families confirmed simple codominant inheritance for allozymes at these loci and also showed that these *Aesculus* species and hybrids are predominantly outcrossing.

Of the 14 enzyme loci examined, 10–12 were polymorphic in most populations, except for the population of *A. parviflora*, which had only three polymorphic loci. In populations of *A. pavia*, *A. sylvatica*, and *A. flava* that showed no morphological evidence of hybridization, 85 electrophoretic alleles were identified, of which about one-third varied significantly in frequency among these species. About 27 alleles were absent or rare in one or two taxa, and thus were useful as partially diagnostic genetic markers of the species possessing the allele (table 2). Unfortunately, none of the species was fixed for alternative alleles, although a few of the loci were nearly so (e.g., *Me2* and *Shk2* in *A. pavia* and *A. sylvatica*).

Suspected hybrid populations between *A. pavia* and *A. sylvatica* possessed all or most of the alleles diagnostic for the hypothesized parental taxa, clearly suggesting relatedness to both species (table 2; dePamphilis and Wyatt 1989). Furthermore, the alleles present in putative hybrid populations were almost entirely a combination of those alleles present in the species populations. Only two alleles were unique to the putative hybrid populations, and both of these were rare (dePamphilis and Wyatt 1989). Genetic admixture proportions, which estimate the proportion of genes contributed by parental populations, showed that most putative hybrid populations possess a substantial fraction of genes from the suspected parents. *Aesculus sylvatica* and *A. flava* were found to be allozymically more similar to each other than either is to *A. pavia*. Suspected hybrid populations involving *A. sylvatica* and *A. flava* generally shared higher genetic identities with *A. sylvatica*, but these populations did possess the relatively few marker alleles of both species (table 2). *Aesculus glabra* was also found to be allozymically similar to *A. flava*, but it could be excluded from the admixture analysis based on morphological grounds. On the other hand, *A. parviflora* was allozymically very distinct from all other species and their hybrids.

To investigate in detail the individual variation seen in *A. pavia*, *A. sylvatica*, and putative hybrid populations between them, computer simulations of random mating were used to generate artificial parental, F_1 hybrid, and backcross individuals, based on the allele frequencies of populations outside the suspected hybrid zone (dePamphilis 1988a). The multilocus genotypes of actual and simulated individuals were then compared using a character index (CI) scoring system (table 3; Sage and Selander 1979) similar to Anderson's (1949) hybrid index. These analyses showed that the distributions of individual CI scores observed in populations not suspected of hybridization were nearly the same as that for the simulated individuals generated under random mating within each species (dePamphilis and Wyatt 1989). Suspected hybrid populations, and a few populations beyond the morphological hybrid zone, included individuals that were unambiguously identifiable as hybrids (table 3; dePamphilis and Wyatt 1989). Some hybrid populations were observed more than 100 km into the Piedmont, suggesting the possibility of very long-distance gene flow. Un-

fortunately, although the amount of allozyme differentiation between *A. pavia* and *A. sylvatica* was about the same as that typical of congeneric plant species (Crawford 1985), differentiation between them proved insufficient to permit discrimination of F_1 hybrids from backcross individuals. Thus, while the patterns of character index scores were compatible with the possibility of introgression, these data could not be used to identify introgressive individuals with complete confidence.

In every population in which hybrids could be unambiguously identified, at least one of the suspected parents was missing at that location. A few hybrid plants were observed in populations below the Fall Line, which delimits the Piedmont and Coastal Plain, but these always lacked individuals identifiable as *A. sylvatica*. Hybrid populations were observed at distances up to 150 km above the Fall Line, and these distant hybrid populations always lacked individuals identifiable as *A. pavia*. Three populations (nos. 16, 20, and 24 of fig. 1 and table 2) consisted primarily of hybrid plants, with no individuals that could be unambiguously identified as either *A. pavia* or *A. sylvatica*.

In summary, patterns of morphological and allozymic variation together strongly suggest the existence of a broad hybrid zone involving *A. pavia*, *A. sylvatica*, and *A. flava* in the southeastern United States. Detailed studies further indicate a strongly asymmetrical pattern of hybrid occurrence, and presumably of gene flow, biased toward *A. sylvatica*, the common species in the Piedmont. The great width of the zone, its asymmetry, and the fact that hybrid populations typically appear to be missing one of the parents suggest a predominantly directional pattern of gene flow that may cover large distances. Additional information from distribution patterns, reports of meiotic irregularities in putative hybrids, and the observation of spontaneous hybrids between cultivated plants provide support for the hypothesis of hybridization. Further support could be obtained from quantitative comparisons of meiotic irregularities or pollen viabilities in suspected hybrid and non-hybrid populations and from experimental evidence of crossability between the species. Furthermore, a lack of knowledge about the possible mechanism(s) of gene flow, both of pollen and seeds, and of the survival and fitness of hybrids in nature, limits the depth of our understanding of how the hybrid zone may have formed or is currently maintained.

Methods

Morphology. To examine patterns of morphological variation in *A. pavia*, *A. sylvatica*, *A. flava*, and putative hybrid populations, Hardin's (1957d, p. 196) data were recoded for statistical analysis. Measurements of two continuous variables, stamen length and calyx length (both to the nearest mm), were used directly. Three ordinal variables were scored as follows: pedicel and calyx glandularity, 1 = glandless, 2 = few glands, and 3 = glandular; perianth color, 1 = yellow, 2 = yellow-red, and 3 = red; petal margin hairs, 1 = villous, 2 = glandular-villous, 3 = glandular. Three nominal variables were scored as follows: stature, 1 = shrub, 2 = large tree; petiolule length, 1 = >3 mm, 2 = <3 mm; stamen relative length, 1 = < lateral petal, 2 = > lateral petal. A population "mean" and "coefficient of variation" were calculated for each variable. Principal components analysis (PCA) (Morrison 1976; Neff and Smith 1979), based on the population means for each of the eight variables, was used to describe overall patterns of morphological variation. Calculations were performed using the Statistical Analysis System (SAS Institute 1985).

Pollen Viability. Two methods were used to estimate pollen viability for plants from putative hybrid and non-hybrid populations: 1) in vitro germination using the hanging drop technique (Mortenson et al. 1964) and 2) pollen stainability (Radford et al. 1974). Flowers were collected in mid-morning from plants in field populations and were transported in a shaded open box to the laboratory for immediate analysis. For populations more than 30 minutes from the laboratory, branches bearing inflorescences were collected and the cut branches were placed in water for transport to the laboratory. From at least two flowers on each plant, a dehisced anther with visible pollen was touched to a 5 μl drop of germination medium placed on a cover slip. The medium consisted of 22% sucrose (w/v), 1.062 g/liter $Ca(NO_3)_2$, 0.909 g/liter KNO_3, and 1.107 g/liter $MgSO_4$. The cover slip was inverted over a depression slide, and the slide was placed in a humid box at 22°C.

Pollen grains typically began germinating within 20 minutes, but 3 hours were allowed

for full germination. At that time, a drop of aniline blue stain was added (0.1 g aniline blue, 50 ml lactic acid, 100 ml H_2O) and permitted to stain for at least 30 minutes. Grains were scored as germinated if a pollen tube equal in length to at least the diameter of the grain was observed. Non-germinated grains were then classified as darkly stained or only lightly stained. At least two counts of 200 grains were obtained from separate flowers of each plant. Initial trials showed that fresh pollen from flowers that had been open to pollinator activity varied greatly in both stainability and germinability, presumably due to the removal of early-shedding viable grains by pollen-collecting floral visitors (dePamphilis, unpubl. data). To reduce this source of variation, pollen for these observations was obtained from flowers enclosed in bridal-veil bags prior to anthesis.

Two additional experiments were performed in 1985 to estimate the lifespan of pollen under field conditions. In both cases, pollen was obtained from inflorescences enclosed in bridal-veil bags. From the *A. sylvatica* population in Clarke Co., Georgia, we collected flowers at various times following anthesis until the flowers began to senesce (about 3 days). For *A. pavia*, we attempted to assess the potential lifespan of pollen on ruby-throated hummingbirds (*Archilochus colubris*), using non-living, but intact, specimens that had been stored in a freezer since death (Dr. H. C. Yeatman, pers. comm.). Inflorescence-bearing branches were collected from a cultivated population growing in Clarke Co., Georgia, and transported to a screen-enclosed porch. Specimens were brought into contact with the flowers in a fashion similar to foraging by living birds and allowed to accumulate pollen on the feathers around the base of the bill. The birds were brought into contact only with fresh flowers with yellow banner petals. Pollen was then periodically removed from the birds with a fine brush and assayed for germinability as above. Because of the relatively small number of pollen grains that could be recovered from the birds, the experiment was terminated after 24 hours.

Crossing Experiments. To assess the potential for interspecific hybridization, a crossing experiment was performed in the spring of 1982 that involved two populations each of *A. sylvatica* (both in Clarke Co., Georgia) and *A. pavia* (cultivated populations in Clarke and Oglethorpe cos., Georgia). Previous studies had suggested that natural levels of seed-set are very low in *Aesculus* (Benseler 1975; Bertin 1982a; Hardin 1955), so experiments were designed to try to increase the chances of obtaining some viable fruit production. Inflorescences were enclosed in bridal-veil bags with thin wire frames. The andromonoecious breeding system of *Aesculus* is heavily biased toward the production of staminate flowers (Benseler 1975; Bertin 1982a; Coker and Totten 1945; Hardin 1955), so perfect flowers had to be located by examination of a large number of unopened, expanding flower buds using blunt dissecting tools to push aside the calyx lobes. Perfect flowers were emasculated approximately 1 day prior to anthesis. On the following day, when the flower opened, it was subjected to one of three treatments: 1) pollination with pollen from different individuals of the same species, 2) pollination with pollen from the other species, or 3) no pollination (control). Each pollination was performed twice, first on the day the emasculated flower opened, and again 2 days later, just prior to senescence. A fresh anther with abundant visible pollen was rubbed on the distal 5 mm and tip of the style. To decrease the likelihood of pollen donor limitation of seed-set, pollen from two different pollen donors was used for each pollination. Survivorship of the pollinated flowers was recorded every 10 days after the first pollination.

Pollination Biology. To examine the potential for interspecific gene flow via pollen movement, studies were made of flowering phenology and pollinator behavior in parental as well as hybrid populations.

Pollinator observations were conducted over the period 1981–1986 in natural populations of *A. pavia* (Appling and Burke cos., Georgia; Tuscaloosa Co., Alabama), *A. sylvatica* (Clarke and Oconee cos., Georgia), *A. flava* (Rabun Co., Georgia; Buncombe Co., North Carolina), and putative *A. sylvatica* × *pavia* populations (DeKalb, Douglas and Henry cos. Georgia). Except for the population from Clarke Co., Georgia, each of these populations was included in the electrophoretic survey described above (dePamphilis 1988a; dePamphilis and Wyatt 1989). Within each population, floral visitors to *Aesculus* were observed for 45-minute periods distributed throughout the day during the peak period of flowering. Most observations were made from

TABLE 4. Two estimates of pollen viability for *Aesculus* species and hybrid populations. Estimates were made on open flowers with yellow banner petals after enclosure in bridal-veil bags for 2 days. *N* is number of individuals. Abbreviations are first letter of specific epithet. [1] Cultivated population.

Taxon	Population	*N*	Stainability (%) mean (range)	Germinability (%) mean (range)
A. pavia	Clarke Co., Georgia[1]	4	98.2 (96.5–99.4)	92.0 (83.8–95.2)
s × *p*	Henry Co., Georgia	23	94.0 (77.8–100)	72.6 (0.0–95.2)
	Bartow Co., Georgia	2	60.6 (45.2–76.2)	37.0 (35.4–38.6)
	Dekalb Co., Georgia	9	95.2 (78.5–99.8)	92.1 (73.0–98.9)
A. sylvatica	Clarke Co., Georgia	9	94.2 (81.8–99.2)	83.8 (68.7–97.6)
	Clarke Co., Georgia	2	97.1 (95.0–99.3)	93.9 (93.4–94.5)
f × (*s* × *p*)	Gordon Co., Georgia	6	92.2 (79.4–97.6)	60.7 (14.1–93.1)
A. flava	Buncombe Co., North Carolina	15	95.6 (82.6–99.2)	86.6 (75.5–95.1)

two different stationary locations over two or more years. Additional nighttime observations and observations early and late in the flowering season were added when possible. Additional details of the pollinator observations, as well as levels of fruit-set and seed viability, will be described by dePamphilis and Wyatt (in prep.). The identity, frequency of visitation, and behavior of floral visitors were noted, with particular reference to whether the visitor was collecting pollen and/or nectar and whether the visitor was judged able to effect pollination. Voucher specimens of all insect species have been deposited in the Natural History Museum at the University of Georgia.

Flowering phenology was scored from herbarium specimens of *A. pavia*, *A. sylvatica*, *A. flava*, and their hybrids from localities in Georgia and adjacent counties of North Carolina and Tennessee. Phenology was also studied for *A. parviflora* across its range in Alabama and Georgia (Wyatt 1985). Flowering specimens were identified using the morphological criteria of Hardin (1957b, 1957d; table 1). Only specimens that could be assigned an exact date and county location were used. For each species and hybrid group, flowering dates were converted to their respective days of the year and the mean and range were calculated. In addition, the method of Bertin (1982b), which takes into consideration the phenological development of each specimen, was used to calculate the "peak" flowering date for each group. Each specimen was given a score representing its phenological stage, based on the proportion of flowers unopened on the inflorescence: 2 = early flowering (66.7–99.9% still in bud), 3 = middle flowering (33.4–66.6% still in bud), and 4 = late flowering (0–33.3% still in bud). The "peak" flowering date was then estimated by calculating the expected date at which the average score would be 3.0 (Bertin 1982b).

RESULTS

Pollen Viability. Pollen viability, assayed both by germination percentages and stainability, was generally high, but some variability was observed (table 4). Plants from hybrid populations had significantly lower pollen germination percentages than did plants from nonhybrid populations ($F = 5.95$; d.f. = 1,36; $P < 0.02$). All of the plants with <60% pollen germination were in hybrid populations (9 of 40 plants assayed). Germination rates among hybrid populations were not uniformly depressed, however, as the only significant difference among populations was for Bartow Co., Georgia, vs. the other populations (GT2 test following ANOVA; Sokal and Rohlf 1981). In contrast to the results for pollen germinability, pollen stainability did not differ significantly between plants from hybrid and non-hybrid populations ($F = 0.99$; d.f. = 1,36; $P < 0.33$). Only one plant with highly inviable pollen was observed, and it was also from Bartow Co., Georgia, with 45.2% stainability.

Pollen germination percentages for *A. sylvatica* were slightly greater 72 hours after dehiscence, but by 96 hours after dehiscence, germinability declined significantly to about 60% of that seen in fresh pollen (table 5). When pollen of *A. pavia* was sampled from hummingbird specimens, a more variable situation was

TABLE 5. In vitro germination percentages for pollen of *A. pavia* and *A. sylvatica*. All values are scaled to time 0 = 100%. Germination for *A. pavia* was determined from pollen on hummingbird specimens over the course of 24 hours (see text). Germination for *A. sylvatica* was determined from flowers at various times after dehiscence. Each value represents the mean of three flowers, with at least 200 pollen grains scored from each flower. [a] Banner petals yellow. [b] Banner petals orange. [c] Banner petals red.

Species	Plant	Hours			
		0[a]	24[a]	72[b]	96[c]
A. pavia	1	100	51.0	—	—
	2	100	136.0	—	—
	3	100	174.0	—	—
A. sylvatica	1	100	—	108.0	47.4
	2	100	—	112.0	81.0
	3	100	—	102.0	56.8

observed after just 24 hours. Three trials gave germination percentages varying from 51% to 174% of that of fresh pollen sampled from the birds immediately after simulated foraging.

Crossing Experiments. Only six fruits were produced following the experimental pollinations, all by plants of *A. pavia* (table 6). These included two from intraspecific pollinations and four from interspecific pollinations. Although similar effort was expended locating perfect flowers in both species, more perfect flowers were produced by *A. pavia*, thus imbalancing the actual number of crosses performed. None of the much smaller number of crosses to *A. sylvatica* produced a fruit, nor did the control flowers from either species, which were emasculated and not pollinated. Survivorship patterns for the flowers in all treatments were similar for the first 10 or 20 days, but young fruits remaining 40 days after flowering generally survived until maturity in early September. Each fruit produced in the experiment contained a single small, but apparently viable, seed that was placed in a sealed plastic bag in a refrigerator for 60 days. At that time, the seeds were planted in potting soil. Unlike all previous experience we have had germinating seeds of these species (e.g., dePamphilis 1988a), none of the seeds germinated.

Pollination Biology. A variety of floral visitors was observed for each of the *Aesculus* species and hybrids, only some of which were judged to be effective as pollinators (table 7). In all populations studied, bumblebees (*Bombus* spp.) were the most common visitors. These insects foraged for both pollen and nectar and were judged able to effect pollination in the process. Both the stigmas of perfect flowers and anthers of the more numerous staminate flowers are held at about the same position and were contacted by floral visitors that collected pollen or visited flowers for nectar. The stigma and distal portion of the style of freshly opened flowers were usually covered with orange *Aesculus* pollen by mid-morning. Other visitors also judged to be effective as pollinators included several species of andrenid and anthophorid bees (collecting pollen and nectar), butterflies (collecting nectar), and ruby-throated hummingbirds (*Archilochus colubris*). The latter were typically observed late in the flowering season of each species and were always much less common than the bumblebees. Pollen accumulated on feathers near and around the base of the bill while the birds foraged for nectar in the tubular flowers. The orange pollen, which could often be seen on the birds from a considerable distance, would then be deposited on the stigmas as an incidental by-product of these activities.

Several flower visitors foraged only for nectar through holes or slits made in the calyx (*Xy-*

TABLE 6. Results of crossing experiments involving *A. pavia* and *A sylvatica*. Pistillate parent given first.

Treatment	*N*	Number surviving							
		10 d	20 d	30 d	40 d	50 d	60 d	70 d	120 d
A. pavia × *pavia*	58	50	35	6	2	2	2	2	2
A. sylvatica × *pavia*	7	5	1						
A. pavia × *sylvatica*	57	50	37	7	4	4	4	4	4
A. sylvatica × *sylvatica*	6	6	3	1	1				
A. pavia, not pollinated	54	47	35	3					
A. sylvatica, not pollinated	6	5	1						

TABLE 7. Common flower visitors of *Aesculus* species and putative hybrid populations between *A. sylvatica* and *A. pavia*. A plus sign ("+") indicates an effective pollinator, whereas a minus sign ("−") indicates a visitor ineffective in pollination. Abbreviations are first letter of specific epithet.

Floral visitor	*Aesculus* species or hybrid			
	p	*s* × *p*	*s*	*f*
Apodiformes: Trochilidae				
Archilochus colubris (L.)	+	+	+	+
Hymenoptera: Andrenidae				
Andrena sp.				+
Hymenoptera: Anthophoridae				
Emphoropsis laboriosa Fabr.	+/−			
Synhalonia atriventris Smith	+	+	+	
Xylocopa virginica L.	−	−	−	
Hymenoptera: Apidae				
Apis mellifera L.	−	−	−	
Bombus affinis Cresson				+
B. bimaculatus Cresson	+	+	+	+
B. griseocollis DeGeer	−			
B. impatiens Cresson	+/−			
B. pennsylvanicus DeGeer	+	+	+	+
B. vagans Smith				+
Lepidoptera: Hesperidae				
Erynnis horatius Scudder and Burgess	+	+		
Lepidoptera: Papilionidae				
Papilio glaucus L.		+		

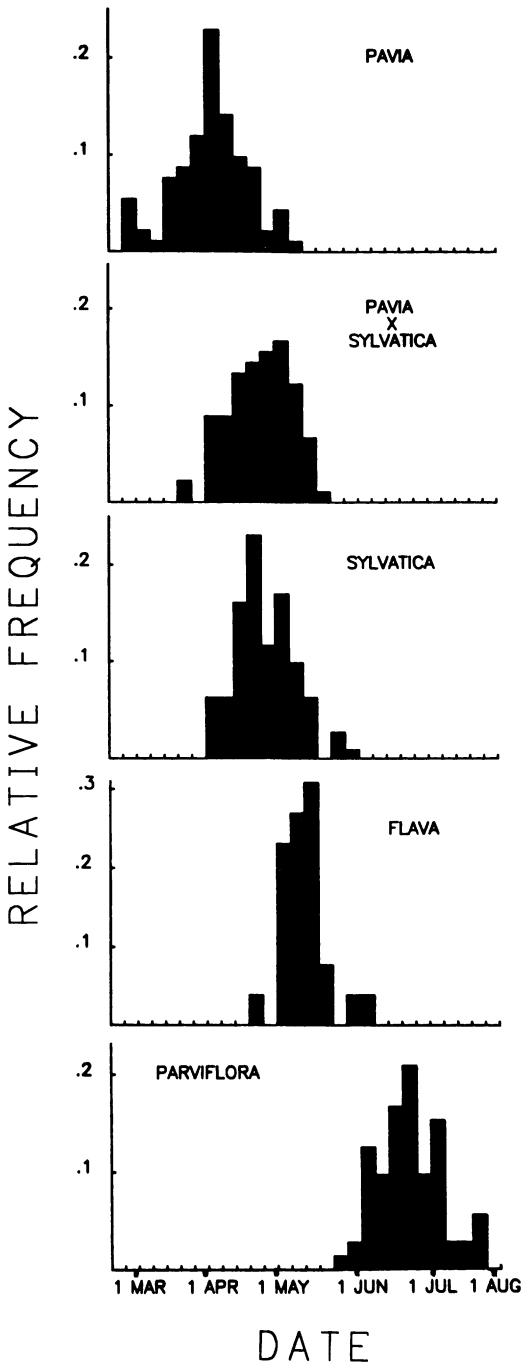

FIG. 3. Relative frequency histograms of collection dates for flowering specimens of *Aesculus* species and putative hybrids from Georgia and adjacent counties of North Carolina and Tennessee. Plants were identified using criteria of Hardin (1957b, 1957d), as summarized in table 1. *Aesculus parviflora* dates are for plants growing in Alabama and Georgia.

locopa virginica, Bombus griseocollis, Apis mellifera), and these insects were obviously ineffective as pollinators. A few species (*Emphoropsis laboriosa* and *Bombus impatiens*) were sometimes observed robbing nectar through holes in the calyx and sometimes visiting flowers "legitimately," such that both anthers and stigmas would be contacted and pollination could occur. Further details of flower visitor frequency, behavior, and foraging activities on and among plants within populations will be presented in a later paper (dePamphilis and Wyatt, in prep.).

Although some of the pollinators were restricted to only one of the *Aesculus* species, a number were observed in populations of two or more species. The small bee, *Synhalonia atriventris*, was commonly observed in populations of *A. pavia*, *A. sylvatica* and hybrid populations,

TABLE 8. Mean and peak flowering dates for *Aesculus* species and hybrids, based on herbarium specimens. Identifications follow Hardin (1957b, 1957d; see table 1). Area surveyed is Georgia and Alabama for *A. parviflora*, Georgia and adjacent counties of southwestern North Carolina for *A. flava*, and Georgia for all other taxa. *N* is number of individual specimens and s.d. is the standard deviation. Mean dates followed by the same small letter do not differ at $P = 0.05$ by GT2 test, following analysis of variance (Sokal and Rohlf 1981). [1] Insufficient sample size for calculation of peak flowering date.

Taxon	*N*	Mean date	s.d.	Peak date
A. pavia	92	3 Apr *a*	16.0	29 Mar
A. sylvatica × *pavia*	90	24 Apr *b*	13.9	11 Apr
A. sylvatica	112	22 Apr *b*	12.5	19 Apr
A. sylvatica × *flava*	17	30 Apr *b, c*	13.7	22 Apr
A. flava × (*sylvatica* × *pavia*)	8	3 May *b, c*	10.6	—[1]
A. flava	26	11 May *c*	9.5	4 May
A. parviflora	72	26 Jun *d*	14.0	28 Jun

but not in populations of *A. flava*. Two species of bumblebee (*B. bimaculatus* and *B. pennsylvanicus*) and the ruby-throated hummingbird visited all three species and hybrids.

The flowering season for *Aesculus* in Georgia and adjacent states begins in late February with *A. pavia* and continues without interruption through the end of July with the late-flowering *A. parviflora* (fig. 3). In part, this represents a progression of flowering from *A. pavia* in the southern Coastal Plain, through *A. sylvatica* in the Piedmont, to *A. flava*, a species of the Appalachian Mountains. *Aesculus parviflora*, however, frequently occurs sympatrically with *A. pavia* in the Coastal Plain, but flowers much later. Although the mean dates of flowering for each species differ significantly (table 8), there is considerable overlap in flowering, particularly between *A. pavia*, *A. sylvatica*, and *A. flava* (fig. 3). Interestingly, flowering dates for hybrid specimens, particularly those identified as *A. sylvatica* × *pavia*, were all similar to that of *A. sylvatica* (table 8). Hybrids involving *A. flava* were shifted toward the later flowering dates observed for that species.

DISCUSSION

The distribution of genetic variability within and among populations of *Aesculus* has shown clearly that the putative hybrid populations identified by Hardin (1957c, 1957d; table 2) cannot be explained as single species or as mixtures of pure species without hybridization. Most of the hybrid populations were predominantly *A. sylvatica*, but substantial proportions of alleles characteristic of *A. pavia* and sometimes of *A. flava* were observed. Within the hybrid zone involving *A. pavia* and *A. sylvatica*, individual plants with combinations of alleles of both species were unambiguously identified as hybrids in all but one of the putative hybrid populations studied. Unambiguous hybrids were also detected in several nearby populations that showed no morphological evidence of hybridization (dePamphilis and Wyatt 1989). Thus, the hybrid zone, defined genetically, is somewhat larger than expected on the basis of morphology alone (fig. 1).

Another interpretation, and one that cannot be rejected using the electrophoretic data alone, is that the putative hybrid populations are remnants of an ancestral gene pool that gave rise to the entities presently interpreted as parental species (Heiser 1973). In the absence of a distinctly unidirectional event, such as a unique gene duplication or polyploidy (Gottlieb 1972), it is difficult to distinguish between secondary zones of hybridization (i.e., hybridization following secondary contact) versus primary zones (i.e., remnant populations) based on studies of genetic markers alone (Endler 1977). Because the populations in question form coincident clines in allele frequency at several loci (dePamphilis and Wyatt 1989), a zone of secondary contact is more likely (Barton and Hewitt 1985). In addition, the overriding impression from the electrophoretic data is that the hybrid zone represents very nearly the sum of several genomes and not a separate entity. If *A. pavia* and *A. sylvatica* had been derived from the zone, it would be reasonable to expect a similar amount of genetic divergence between all three entities: *A. pavia*, *A. sylvatica*, and the zone between them.

This is not the case, as both *A. pavia* and *A. sylvatica* have several unique alleles at high frequencies and unique alleles are lacking in the zone except for two in very low frequency (dePamphilis and Wyatt 1989). More importantly, previous observations of meiotic irregularities in putative hybrid plants and data presented in this paper of decreased pollen germinability in plants from putative hybrid populations argue that this is indeed a secondary contact zone. Such irregularities would not be expected if the zone actually consisted of remnant populations, but they are frequently associated with hybridization in plants (Grant 1981; Stebbins 1950).

Although none of the experimental crosses produced viable offspring, these results do not invalidate observations of earlier workers (see Hardin 1957d), who found that these species cross readily when grown under common garden conditions. Previous experience with these species suggested that seed-set would be low (Bertin 1982a; Hardin 1955), and the levels observed in this study are not unusual. The results of the crossing experiments do, at any rate, suggest that fruit-set is not greatly reduced in interspecific crosses. Due to the extremely time-consuming nature of performing experimental crosses with *Aesculus*, this will probably not be an important source of information unless conditions can be discovered that ensure higher levels of seed-set.

It has often been stated that hybridization in plants is very localized, with hybrids surviving primarily in disturbed habitats unsuited for the parental species (Anderson 1949, 1953; Heiser 1973; Wagner 1969, 1970). Survival of hybrids is often thought to be possible mainly through special mechanisms such as cloning, apomixis, and self-fertilization (Grant 1981; Heiser 1973; Stebbins 1950). None of these generalizations appear to be valid for *Aesculus*, where hybridization is widespread throughout a very large hybrid zone that is, in places, >200 km wide. Hybrid populations do frequently occur at locations with a history of disturbance, such as logging and nearby roadcuts, but these are no more frequent or severe than those observed in populations of the parental species (dePamphilis, unpubl. data). It is possible that the Fall Line, a location of fairly rapid change in elevation from the upper Coastal Plain (at around 75 m) to the lower Piedmont (around 225 m), actually represents a natural ecotonal area where the parentals are at a disadvantage. Clonal development, which is extremely limited in the parental species (dePamphilis 1988a), is rare in the hybrids as well, judging by the very high levels of genotypic diversity observed in hybrid populations (dePamphilis and Wyatt 1989). There is no evidence of apomictic seed production in these species or hybrids, which have mean outcrossing rates averaging at least 80% (dePamphilis 1988a). Finally, other than a somewhat decreased pollen viability, hybrids appear to suffer from no obvious reproductive disadvantage relative to parentals, judging from similar levels of pollinator attraction and viable seed production (dePamphilis and Wyatt, in prep.). All of the available evidence suggests that hybrids occur in a variety of habitats similar to those of the parental species and that their maintenance in these populations is through normal sexual reproduction.

It is also true that most land in the Piedmont was under cultivation as recently as the early 1900's (Nelson 1957). While agriculture undoubtedly influenced current forest composition (Brender 1974; Nelson 1957), these historical patterns of disturbance are unlikely to have promoted hybridization between species of *Aesculus*. This is hypothesized because neither forest cutting nor cultivation would serve to bring the parental species into closer proximity or create habitats for which hybrids are particularly well-suited. We suspect that both the parental species and the hybrids survived the intense land-use activities of the eighteenth and nineteenth centuries by occupying habitats of marginal value for cultivation and have since expanded locally into the recovering forests. The frequent occurrence of *Aesculus* populations on rocky slopes, along streams, and near shallow-soiled rock outcrops of the lower Piedmont (dePamphilis and Wyatt, unpubl. data) are consistent with this view.

Given that extensive hybridization appears the most likely explanation for patterns of both morphological and electrophoretic variation, can the observations from pollination biology help to explain why hybridization is so widespread? The mean and peak flowering dates for each of the species do differ, but the distributions of flowering dates indicate substantial overlap.

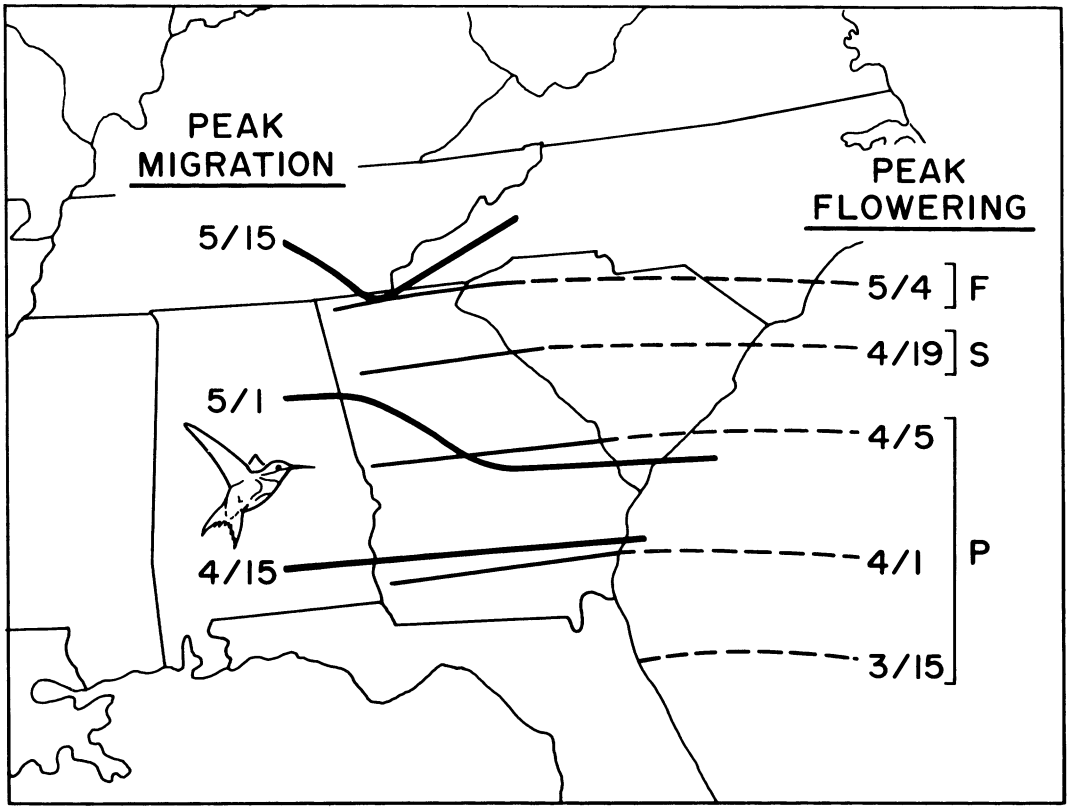

FIG. 4. Peak migration dates for the ruby-throated hummingbird (*Archilochus colubris*) and dates of peak flowering for *Aesculus* species in Georgia and adjacent counties of North Carolina. Migration dates and flowering peaks for *A. pavia* are redrawn from Bertin (1982b), while flowering peaks for *A. sylvatica* and *A. flava* were calculated from herbarium specimens (table 7) using the method of Bertin (1982b).

Often two or more species of *Aesculus* and their hybrids share pollinators, including bumblebees, a small bee (*Synhalonia atriventris*), and the ruby-throated hummingbird. A small skipper butterfly (*Erynnis horatius*) was also observed in populations of *A. pavia* and *A. sylvatica* × *pavia*. Most of these animals are unlikely to travel very great distances. Queen bumblebees emerge from hibernation early in the spring and spend a period of up to two weeks or longer searching for an appropriate nest site (Heinrich 1979). Although these "wandering" movements may cover a distance of several kilometers, very long-distance flights probably do not occur (R. W. Matthews, pers. comm.). Similarly, adults of the skipper *Erynnis horatius*, which is widespread throughout Georgia (Harris 1972), emerge in the spring (Burns 1964) and search for appropriate locations to oviposit. Again, movements are probably over short distances. We have no information concerning long-distance flights of *Synhalonia atriventris*.

In contrast, ruby-throated hummingbirds are well-known for directed spring migratory flights that cover great distances. Most ruby-throats leave the Yucatan Peninsula of Mexico in late winter and fly non-stop some 800 km over the Gulf of Mexico to points on the Gulf Coast including Louisiana and Alabama (Greenewalt 1960; S. Gauthreaux, pers. comm.). Another smaller population overwinters in the Caribbean and migrates north from Florida. By late March, both groups are actively migrating overland to their eventual nesting sites, which encompass most of eastern North America, including sites as far north as Canada. Bertin

(1982b) compiled extensive records of spring and fall migration dates for these birds and peak flowering dates for a number of plants on which ruby-throats forage, including *A. pavia* (fig. 4). To these data, we have added similar estimates of peak flowering dates for *A. sylvatica* and *A. flava* in Georgia and adjacent counties of North Carolina (table 8, fig. 4). As the birds migrate northward, there is a similar progression in flowering dates for each of the *Aesculus* species, with flowering peaks about 10–14 days ahead of peak migration dates.

If a migrating hummingbird were to pause to forage on *A. pavia* and then continue migrating northward, how far might it travel with viable pollen? A full answer to this question would involve knowledge of the dynamics of *Aesculus* pollen on migrating ruby-throats, for the pollen could fall off, be replaced by pollen from other plants, or be groomed from the feathers. Nevertheless, because birds bearing the orange pollen of *Aesculus* are readily observed in the field, it is likely that some pollen remains on birds as they travel northward. Evidence from this study suggests that pollen viability of *Aesculus* is not greatly decreased under field conditions for at least one day after anther dehiscence and possibly for 3 days or more. Measured flight rates for ruby-throats show that the birds maintain at least 30 km/hr over long distances and closer to 60–70 km/hr over shorter distances (Meinhertzhagen 1955). These figures would suggest that in a short time relative to the flight over the Gulf, a migrating bird carrying viable pollen could easily travel a distance equal to the width of the hybrid zone.

Perhaps even more startling than the relationship between migration dates and flowering peaks is the general concentration of spring-migrating birds, including hummingbirds, along major river courses (S. Sibley, pers. comm.). These routes may offer easy navigation and, if the birds do not track the river too closely, a reasonably straight course. Hardin (1957c, p. 50) stated,

> Recent studies of other populations in Georgia and hundreds of herbarium sheets show that *A. pavia*, or at least *A. sylvatica* with a very strong influence of *A. pavia*, extends up the rivers from the Coastal Plain well into the Piedmont. Hybrid populations, for example, are common along the Savannah River far up into northwestern South Carolina. Also the Chattahoochee, Oconee, Ocmulgee and Yellow Rivers, among others, have served as routes for this apparent gene flow.

Hardin (1957c) argued that this pattern of hybrid plants distributed far from *A. pavia* suggested that this species was once present in the Piedmont, possibly during a period when the Coastal Plain was submerged. We suggest that it is also possible that *A. pavia* never existed sympatrically with *A. sylvatica* in the Piedmont, but rather that hybrids along river courses far from parental populations of *A. pavia* reflect precisely the distribution pattern that would be predicted if migrating hummingbirds acted as vectors of long-distance pollen movement.

Dispersal by seeds, another possible means of dispersal that could lead to hybridization, is probably limited to relatively short distances. Seeds of the eastern North American species of *Aesculus* are among the largest of North American seeds (Schopmeyer 1974). With the exception of *A. glabra*, whose spiny capsules could conceivably attach to the fur of a large mammal, these seeds lack any obvious mechanism of dispersal other than gravity. Dispersal of intact seeds probably occurs to some extent by rodents, which often stash and consume the seeds (Levy 1984; dePamphilis, pers. obs.). We have observed seeds of *A. flava* in streams and at the water's edge on occasion 10–30 m from the nearest seed-bearing trees (dePamphilis, pers. obs.). How long seeds remain viable in water and how far viable seeds may travel is unknown. Nevertheless, the possibility that some viable seeds travel downstream cannot be discounted. Because the predominant distribution of hybrids involving *A. flava* is at elevations below the usual distribution of *A. flava* in the upper Piedmont (fig. 1), this mode of seed transport deserves further investigation.

The hypothesis that long-distance gene flow in *Aesculus* has occurred via hummingbird-mediated pollen dispersal has significance with respect to the way we view hybrid population systems. The allozyme differentiation between *A. pavia* and *A. sylvatica* is not sufficient, with the present sample of gene loci, to identify introgressive individuals with complete certainty. Yet, the allozyme variation, morphological variation, and observations of reproductive be-

havior of these species and hybrids (dePamphilis and Wyatt, in prep.) do strongly suggest that introgression has occurred over a large geographical region. Traditionally, a distinction has been made between "localized" and "dispersed" introgression (Heiser 1973), referring to whether hybrids and presumed introgressants occur only among sympatric species in localized areas or whether introgression occurs between allopatric species. The widespread occurrence of hybrids and presumably of introgressants at great distance from *A. pavia* could be considered, under this definition, an example of dispersed introgression. However, hybridization due to long-distance pollen dispersal followed by localized introgression may provide the most accurate description of how this zone developed. It could prove difficult to distinguish this particular form of "localized" introgression from a true case of "dispersed" introgression.

The hypothesis is also at least partially testable. Organellar genomes, specifically chloroplast DNA and mitochondrial DNA, are inherited strictly through the maternal parent in most angiosperms that have been investigated (Palmer 1985). This mode of inheritance is in contrast to that of most soluble proteins used in allozyme analyses, which are encoded by nuclear DNA and biparentally inherited (Gottlieb 1981; Weeden and Wendel 1989). Thus, hybridization between plants with different organellar DNA markers should result in only the genomic markers of the maternal parent being observed in hybrids or seeds produced by hybrids (Erickson et al. 1983; Palmer 1985). If the broad hybrid zone between *A. sylvatica* and *A. pavia* is a result of the movement of pollen, then maternally inherited genetic markers of *A. pavia* should be absent from the hybrid zone. On the other hand, if gene flow mediated by seed movement of *A. pavia* has had any role in the origin of the hybrid zone, then maternally inherited markers of *A. pavia* should be present in hybrid populations. This would include the situation proposed by Hardin (1957c), in which hybrid populations were considered possible remnants of a past zone of sympatric hybridization, and any case where hybrids had originated because of seeds moved by man or other means. Therefore, if hybridization involving *A. flava* has been due to downstream seed movement, as we suspect, then maternally inherited genetic markers should be present in hybrid populations involving this species. If maternally inherited genetic markers can be identified for these species of *Aesculus*, the distribution of these markers in populations within the hybrid zone should prove extremely interesting.

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