Am. Midl. Nat. 122:275–280

1989

Genetic Evidence for Reproductive Isolation between Hybridizing Limenitis Butterflies (Lepidoptera: Nymphalidae) in Southwestern New Mexico

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ABSTRACT.—Hybridization has been reported in the literature between *Limenitis* nominal species *arthemis, lorquini* and *weidemeyerii* along their zones of contact. Two of these taxa, *L. arthemis arizonensis* and *L. weidemeyerii angustifascia,* were found in sympatry at Pinos Altos, Grant Co., New Mexico. Natural history observations of these populations were made, and they were examined electrophoretically at 19 presumptive loci for evidence of introgressive hybridization. As adults, both taxa share considerable portions of the microhabitat, including partial overlap in mate location sites used by males. However, four loci were fixed for different alleles, with significantly different allelic frequencies at one additional locus. These taxa are apparently fully reproductively isolated. Hybrids between these taxa reported in the literature are not evidence of an introgressive hybrid zone, as found in contact areas between other *Limenitis* taxa. This contact area may represent the reproductively isolated portion of a ring species.

INTRODUCTION

Many hybrid zones are recognized between parapatric nominal species entities in North American populations of Admiral butterflies in the genus *Limenitis* Fabricius (Remington, 1968; Platt, 1983). Platt and Brower (1968) documented a high frequency of introgressive hybridization between taxa *arthemis* (Drury) and *astyanax* (Fabricius), with no evidence of hybrid inferiority (*compare* Waldbauer *et al.,* 1988), and recognized them as geographic forms of the same species. Hybridization occurs along borders between subspecies of *weidemeyerii* W. H. Edwards and *lorquini* Boisduval (Brown, 1934; Remington, 1968) in Nevada and California; these taxa may prove to be conspecific as well. Scott (1986) and C. L. Remington (pers. comm.) have reported hybridization and possible introgression between *lorquini* and *arthemis* in British Columbia, and Remington (1968) reports hybrids between *weidemeyerii* and *arthemis* in Alberta. Hybrids are also reported between *weidemeyerii* (ssp. *angustifascia* Barnes & Benjamin) and *arthemis* (spp. *arizonensis* W. H. Edwards) where they contact in central and E-central Arizona (Bauer, 1954; Ferris, 1969; Perkins and Garth, 1973). These reports and studies of hybridization between nominal *Limenitis* species indicate the possibility of significant genetic contact, and admit the likelihood of conspecificity among all the parapatric entities (*i.e.,* nominal *arthemis* sspp., *weidemeyerii* sspp. and *lorquini* sspp.).

Recent field work in western New Mexico turned up populations of *Limenitis arthemis arizonensis* and *L. weidemeyerii angustifascia* in microsympatry at Bear Creek, in the upper reaches of the Gila River drainage at Pinos Altos (2150 m), Grant Co. Although this locality is slightly disturbed by human industry (mostly cattle grazing), these populations seem likely to have inhabited this locale for a relatively long time. The populations were analyzed genetically using starch gel electrophoresis in an effort to determine whether evidence of introgression is present in the gene pools of these sympatric entities, as a test of the strength of their apparent reproductive isolation.

MATERIALS AND METHODS

Butterflies were observed and haphazardly netted on 28 and 29 June 1988 in a dry stream bed along a road 2–3 km N of the village of Pinos Altos. Fourteen *angustifascia* (11 males, 3 females) and 15 *arizonensis* (11 males, 4 females) were placed individually into glassine envelopes and stored in plastic bags on wet ice in a cooler, and hand-carried alive on ice to U.C. Davis. There they were frozen at −80 C until electrophoretic analysis in January 1989.

All specimens were examined for traits shown in putative natural hybrids, as reported in Perkins and Garth (1973). These are, from *angustifascia* into *arizonensis*: (1) apical submarginal white spots on the dorsal and/or ventral forewing; (2) extra subbasal patches on the ventral hindwing (up to five total); (3) remnants of the white median band on any wing surface; (4) black dorsal scaling, rather than irridescent green or blue, and (5) white overscaling in the ventral hindwing ground color. From *arizonensis* into *angustifascia,* the character states are reversed.

Electrophoretic protocols followed Ayala *et al.* (1972), with slight modifications as described in Porter and Mattoon (1989). Scoring proceeded as follows: the most common electromorph at each locus in a central California population of *Limenitis lorquini* was used as a standard, and given a score of 100. Electromorph variants were scored relative to their distance (in mm) from the standard. For example, a variant migrating 10 mm further than the standard (toward the cathode) was scored as 110; slower (more anodal) electromorphs had correspondingly lower scores. Electromorphs were assumed to be allelic variants, and are referred to as such throughout this paper. Calculations of all population genetic parameters were performed using the computer program BIOSYS-1 (Swofford and Selander, 1981); and formulae can be found in any standard population genetics text (*e.g.*, Hedrick, 1985).

Nineteen metabolic enzyme loci were scored: adenylate kinase (two loci: AK-1, AK-2; Enzyme Commission number: 2.7.4.7), aldolase (ALDO; 4.1.2.13), fumarase (FUM; 4.2.1.2), glutamic-oxaloacetic transaminase (GOT-1; 2.6.1.1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1.2.1.12), α-glycerophosphate dehydrogenase (α-GPD; 1.1.1.8), hexokinase (HK-1, HK-2; 2.7.1.1), isocitrate dehydrogenase (IDH-1, IDH-2; 1.1.1.42), malate dehydrogenase (MDH-1, MDH-2; 1.1.1.37), malic enzyme (ME-1, ME-2; 1.1.1.40), phosphoglucose isomerase (PGI; 5.3.1.9), phosphoglucomutase (PGM; 2.7.5.1) and superoxide dismutase (SOD-1, SOD-2; 1.15.1.1).

RESULTS

Natural history observations.—*Angustifascia* males ($n \cong 25$) were most commonly encountered in more overgrown areas near and among willows, where open patches typically used as perching sites for mate location were small (<10 m) and patchily distributed. Perching sites in open areas along the edges of willow thickets were occasionally used. Males tended to patrol early in the day (0930 to ~1030, standard time), subsequently settling into perch sites. Two males were seen puddling on damp mud. Females were rarely encountered ($n = 5$), and flew among the inner branches of willow shrubs and adjacent vegetation, alighting often; they were rarely seen to cross open patches.

Arizonensis males ($n \cong 20$) were most commonly encountered in more open, sunny areas (>15 m), often perching on the shady side of isolated small trees or shrubs in the stream bed. Few males were encountered before 1100; these were patrolling very fast, or inconspicuously puddling on damp sand or mud. Males (up to five at a time) tended to congregate around virgin or mating females, and three copulating pairs were encountered (no successful

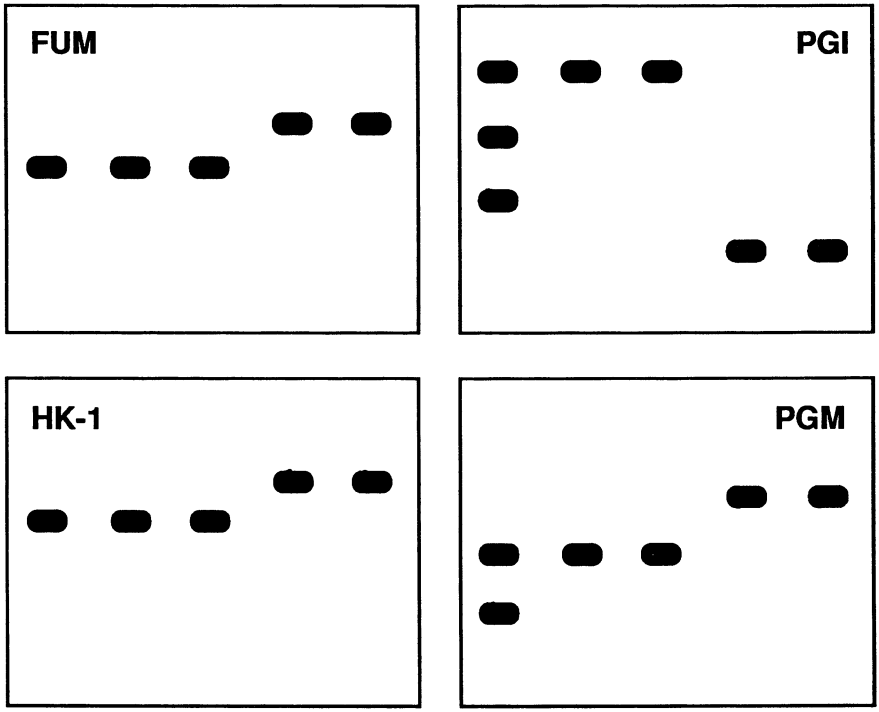

FIG. 1.—Banding patterns for loci FUM, HK-1, PGI, and PGM. From left to right in each: one *lorquini* standard, two *angustifascia,* and two *arizonensis.* Cathode is toward the top of each picture. HK and PGM are monomeric; PGI is dimeric; FUM is tetrameric. Upper left—FUM alleles 100 and 102. Lower left—HK-1 alleles 100 and 105. Upper right—PGI alleles 83, 90 and 100 (one heterozygote). Lower right—PGM alleles 94, 100 and 107 (one heterozygote)

courtships seen) in the late afternoon (1400–1700). Females (n = 6) spent less time flying than males, but did not avoid open areas. One female was observed to flutter among the upper branches of a young poplar tree (*Populus fremontii*; 5 m) for 10 min before laying two eggs.

Habitat overlap was apparent: particular perch sites along the edges of denser vegetation would contain both species over the course of the day. In one encounter, a fresh male *arizonensis* chased a worn male *angustifascia* out of the former's territory. Numerous intra-taxon male-male encounters were observed. Although no intertaxon courtships or copulations were observed, there appeared to be ample opportunity for hybridization to occur.

Wing pattern traits.—The specimens sampled showed no evidence whatsoever of wing pattern introgression.

Electrophoretic analysis.—Allelic frequencies at the nine variable loci are given in Table 1. The remaining 10 loci were fixed for the same allele in both taxa ($AK\text{-}2_{100}$, $ALDO_{100}$, $GAPDH_{100}$, $GOT\text{-}1_{100}$, $\alpha\text{-}GPDH_{100}$, $HK\text{-}2_{100}$, $MDH\text{-}1_{100}$, $ME\text{-}2_{100}$, $SOD\text{-}1_{100}$, $SOD\text{-}2_{100}$). Both taxa shared most common alleles at four variable loci (AK-1, IDH-1, MDH-2, and ME-1). Four additional loci (FUM, HK-1, PGI, and PGM; Fig. 1) shared no alleles between taxa. In χ^2 tests, five loci showed significantly different allelic frequencies between

TABLE 1.—Allelic frequencies at variable loci in *Limenitis arthemis arizonensis* and *L. weidemeyerii angustifascia*

Locus and allele	*ariz.*	*angust.*	Locus and allele	*ariz.*	*angust.*
AK-1			ME-1		
88	0.026		88	0.026	
100	0.974	1.000	93	0.026	
FUM			95	0.026	
100		1.000	100	0.921	1.000
102	1.000		PGI		
HK-1			83	0.895	
100		1.000	88		0.071
105	1.000		90	0.105	
IDH-1			100		0.893
89		0.107	107		0.036
97	0.053		PGM		
100	0.947	0.893	100		0.964
IDH-2			107	0.868	
94	0.026	0.607	109		0.036
100	0.763	0.393	114	0.132	
114	0.211				
MDH-2					
100	0.974	1.000			
107	0.026				

taxa (FUM, HK-1, IDH-2, PGI, and PGM; $P < 0.0001$), with marginally significant differentiation at IDH-1 ($P = 0.061$). No locus showed evidence of deviation from Hardy-Weinberg proportions within taxa. Both taxa showed genetic variability scores (Table 2) at the lower end of the range of most butterfly populations studied.

The genetic distance between taxa, using Nei's (1978) unbiased genetic distance, was 0.26, which converts to an unbiased Nei's identity value of 0.77. This level of differentiation corresponds to well-marked species in typical animal groups (Thorpe, 1983).

DISCUSSION

The lack of evidence for introgression at Pinos Altos suggests that *arizonensis-angustifascia* hybridization events in general are likely to be rare, rather than common, as seen in *arthemis-astyanax* or in *lorquini-weidemeyerii* interactions. Shared alleles at five of the variable loci are as easily explained by common ancestry as by local introgression; the same alleles are shared by *Limenitis arthemis astyanax* and *L. archippus* as well (unpubl. data). This interpretation agrees with an earlier, sketchy report of these taxa in sympatry without apparent hybridization (Gorelick, 1970).

The literature reports of hybridization between *arizonensis* and *angustifascia* are likely to represent rare events, and are probably analogous to the rare hybridizations seen involving *Limenitis archippus* throughout North America (Platt *et al.*, 1978, and references therein). Such hybridization may be explainable by scenarios involving the economics of mate choice (Platt *et al.*, 1978; Wilson and Hedrick, 1982) and/or by immigrants from allopatric populations which are "naive" with regards to appropriate mate recognition behaviors (Bigelow, 1965; Barton and Hewitt, 1985). Under either scenario, a stable hybrid zone will continue to persist with hybrids being produced at low rates, but the data presented here

TABLE 2.—Genetic variability scores for *Limenitis arthemis arizonensis* and *L. weidemeyerii angustifascia*. H_{obs}: observed heterozygosity; H_{exp}: heterozygosity expected from Hardy-Weinberg proportions; P: % polymorphic loci; A: mean alleles per locus. Standard errors are in parentheses

Taxon	H_{obs}	H_{exp}	P	A
arizonensis	0.067 (0.026)	0.065 (0.026)	38.9	1.6 (0.2)
angustifascia	0.056 (0.031)	0.054 (0.030)	11.2	1.3 (0.1)

suggest that such hybridization will not be a significant factor in the evolution of either taxon. However, a significantly superior mutant allele arising in one species may still be able to cross the taxonomic boundary by overcoming hybrid inferiority and genetic drift (Barton, 1979), if backcrossing is possible. Laboratory breeding studies between these two taxa would be interesting in this regard.

Arizoneneis had originally been described as a subspecies of *astyanax* on phenetic grounds before the latter was sunk into *arthemis* by Platt and Brower (1968); *arizonensis* is therefore now classified as a subspecies of *arthemis*. If it is true that *arizonensis* arose as an isolate of *astyanax*, as inferred from the phylogeny suggested in Platt (1983), and if introgressive hybridization between *arthemis* and *weidemeyerii* and/or *lorquini* occurs as reported in western Canada, then the *arizonensis-angustifascia* sympatry represents the reproductively isolated portion of a ring species, sensu Mayr (1963). However, since *arizonensis* is fully allopatric from nominate *astyanax*, *arizonensis* would properly be elevated to species status (Mayr, 1969), even if it did evolve as an isolate of subspecies *astyanax*. Studies of the *Limenitis* populations in western Canada will help resolve these taxonomic questions.

Acknowledgments.—Beth Jakob carried the butterflies back to Davis for me; Art Shapiro froze the bugs and paid for the electrophoresis through his California Agriculture Experiment Station grant *CA-D*-AZO-3944-H, "Range Limitations of Phytophagous Lepidopterans." The gels were run in Brad Shaffer's lab. I was supported by a grant from the Alfred P. Sloan foundation to the Institute of Ecology at U.C. Davis entitled "Cross-disciplinary Studies of Population Structure." All are deeply appreciated.

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SUBMITTED 20 FEBRUARY 1989 ACCEPTED 24 APRIL 1989