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# ESTIMATES OF GENETIC DIFFERENTIATION AMONG CALLOSAMIA SPECIES AND HYALOPHORA CECROPIA (SATURNIIDAE) USING ALLOZYME ELECTROPHORESIS

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**ABSTRACT.** The saturniid silk moths *Callosamia promethea*, *C. angulifera*, and *C. securifera* can be hybridized by hand-pairing but are apparently reproductively isolated in the wild by temporal differences in mating times. Cross-attraction of pheromones among species and the occasional disruption of normal flight and calling rhythms by local weather conditions may result in incomplete reproductive isolation by allochronic mating behavior. Intergeneric hybrids of *Callosamia* and *Hyalophora* also can be produced through handpairing. We performed cellulose acetate electrophoresis of the three *Callosamia* species, *C. angulifera* X *C. promethea* hybrids, and *Hyalophora cecropia* to estimate the amount of genetic differentiation among taxa. Each of the three *Callosamia* species were distinguishable by fixed alleles, an indication that little or no gene flow occurs between the species. Nei's genetic identities between species pairs (calculated across 18 loci) ranged from 0.76 to 0.79, suggesting equal differentiation among the three taxa. The electrophoretic profile *of Hyalophora cecropia* was substantially different; our samples shared alleles with *Callosamia* at only 1 of the 18 loci.

Additional key words: Attacini, reproductive isolation, Callosamia promethea, Callosamia angulifera, Callosamia securifera.

The attacine saturniid silk moths are represented in North America by five genera: Callosamia, Hyalophora, Rothschildia, Samia, and Eupackardia. Although Michener (1952) considered Callosamia a subgenus of Hyalophora, Ferguson (1972) felt the three species represented a discrete group and elevated Callosamia to generic rank. The three Callosamia species share a number of morphological and ecological similarities, but differ in host use. The promethea moth, C. promethea (Drury), is polyphagous on deciduous trees and has a wide geographic range from southern Canada to Florida. The tulip tree silk moth, C. angulifera (Walker), is primarily a specialist on tulip tree (Liriodendron tulipifera L., Magnoliaceae) and co-occurs with southern populations of C. promethea where this host is abundant. The monophagous sweetbay silk moth, C. securifera (Maassen), is restricted to the southeastern coastal plain where its host, sweetbay magnolia (Magnolia virginiana L., Magnoliaceae) grows. Although all three species utilize magnoliaceous hosts, only C. securifera is able to survive on sweetbay

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foliage, which has potent antifeedant and toxic properties towards unadapted insect herbivores (Nitao et al. 1991, 1992).

The three species can be hybridized by hand-pairing but are apparently reproductively isolated in the wild by temporal differences in mating times. In fact, *C. securifera*, which was first described as a variety of *C. angulifera*, only recently was elevated to species status after the temporal isolating mechanism between *Callosamia* species was described (Brown 1972). *Callosamia securifera* females emit pheromone from mid-morning to early afternoon, while *C. promethea* are active from late afternoon to dusk, and *C. angulifera* females do not begin calling until after dusk. There appears to be little or no qualitative difference in the pheromone, as *C. securifera* and *C. angulifera* males can be attracted to calling captive *C. promethea* females (Haskins & Haskins 1958, Peigler 1980, K. S. Johnson unpubl. data).

Hybrids between *Callosamia* species can be obtained by hand-pairing, but hybridization in the wild is believed to be uncommon, since intermediate specimens are rarely collected (Brown 1972, Ferguson 1972, Peigler 1980). Differences in the size of genitalia can prevent successful mating even when moths are hand-paired, as the genitalia of C. promethea are considerably larger than those of the other two species (Peigler 1977). Post-mating incompatabilities contribute to reduced egg hatch, weak larvae, disruption of pupal diapause, and weak or malformed adults (Peigler 1980). Interspecific hybrids involving C. promethea are usually sterile, although a small proportion of C. angulifera X C. securifera hybrids are fertile for three generations (Haskins & Haskins 1958, Peigler 1977, 1980). In addition, differences in host plant use may contribute to post-zygotic selection against hybrid larvae. Neither C. angulifera nor C. promethea can survive on sweetbay magnolia, despite the fact that the former is a near-specialist on another magnoliaceous host, tulip tree, and the latter is highly polyphagous. Understanding the phylogenetic relationships of the Callosamia group would provide a valuable framework for testing hypotheses concerning the evolution of host use and physiological adaptation to host chemistry in this group.

Despite the absence of intermediate specimens in the wild, the crossattraction of the mating pheromone and observations of occasional disruption of normal flight and calling times by local weather conditions raise the possibility that gene flow between the three species may occur. Allozyme electrophoresis has proven to be useful for estimating genetic divergence and phylogenetic relationships of insect taxa at various taxonomic levels (Pashley 1983, Berlocher 1984). We conducted an electrophoretic survey of *C. promethea*, *C. angulifera*, *C. securifera*, *C. promethea* X *C. angulifera* hybrids and *Hyalophora cecropia* allozymes to: 1) evaluate the effectiveness of reproductive isolating mechanisms in

Taxon	Region	Site	Number of Individuals	
C. promethea	Wisconsin	1 Kenosha County	2	
	Michigan	2 Otsego County	2	
	0	3 Barry County	4	
		4 Clinton County	2	
	Maryland	6 Montgomery County	2	
C. angulifera	Michigan	5 Cass County	9	
0.	Virginia	7 Greensville County	3	
C. securifera	North Carolina	8 Bladen County	1	
5	Florida	9 Highlands County, site 1	6	
		10 Highlands county, site 2	4	
		11 Lake County	1	

TABLE 1. Collection locations for *Callosamia* specimens used for allozyme electrophoresis; *C. angulifera* from Cass County, Michigan were taken over a two year period.

*Callosamia*; 2) estimate the relative degree of genetic differentiation among the three species; and 3) compare the genetic distances of *Callosamia* to the closely related *Hyalophora cecropia*.

### MATERIALS AND METHODS

Representatives of the three Callosamia species, C. promethea X C. angulifera hybrids, and Hyalophora cecropia (selected for outgroup comparison) were included in this study. Individuals of Callosamia were field-collected or were offspring of females collected from two to seven sites within their natural geographic ranges. The number of individuals per site ranged from 1 to 6, and because females were needed for other studies during this period, most of the samples for electrophoresis were males. Hybrid C. promethea X C. angulifera came from a semi-natural mating of a captive female C. promethea (collected in southern Wisconsin) with a wild C. angulifera male (Cass County, Michigan). This female released pheromone at dusk and attracted 7 wild C. angulifera males; viable hybrids (n=6) were obtained from one mating. An additional hybrid specimen was the result of a male C. promethea hand-paired with a female C. angulifera. Hyalophora cecropia pupae were collected from several sites in Ingham and Clinton counties, Michigan in 1991. Voucher specimens have been deposited at the museum of the Entomology Department at Michigan State University.

Both adults and pupae were used in allozyme analyses after preliminary studies indicated that there were no appreciable differences in allozyme frequencies between the life stages. Individuals were killed by freezing at -80°C and stored until processing. The posterior half of the abdomen of adult moths and pupae was used for electrophoresis. Tissue was homogenized in 250 ul of extraction buffer (Tris-EDTA-B-mercap-

TABLE 2. Allozymic loci resolved for *Callosamia promethea*, *C. angulifera*, *C. securifera* and *Hyalophora cecropia*, and corresponding running conditions for each enzyme. Buffers and origin positions (an=anode, ce=center, ca=cathode) were selected to keep enzymes centered on the cellulose acetate plates. Asterisks indicate voltage adjusted to maintain current between 9-12 mA per plate.

		Running conditions			
Locus	Enzyme name (E.C. number)	buffer	origin	voltage	time
AAT-1	Aspartate aminotransferase (2.6.1.1)	I	an	275	40
AAT-2					
AC	Acontinase $(4.2.1.3)$	Α	an	٥	40
ACP	Acid phosphatase (3.1.3.2)	$\mathbf{C}$	ce	275	40
ALD	Aldolase (4.1.2.13)	I	ce	275	40
FUM	Fumarase $(4.2.1.2)$	$\mathbf{C}$	ce	275	40
GPI	Glucose phosphate isomerase (5.3.1.9)	I	_	275	40
G6PDH					
HBDH	Hydroxybutyrate dehydrogenase	D	an	300	40
IDH	Isocitrate dehydrogenase (1.1.1.42)	Α	an	٥	40
LDH	Lactate dehydrogenase (1.1.1.27)	в	an	o	40
MDH	Malate dehydrogenase (1.1.1.37)	$\mathbf{C}$	ce	275	40
MPI	Mannose-6-phosphate isomerase (5.3.1.8)	I	an	275	40
<b>P3GDH</b>	3-phosphoglycerate dehydrogenase (1.1.1.95)	$\mathbf{C}$	an	275	40
PEP-LA	Peptidase (leucyl-alanyl) (3.4.11–13)	$\mathbf{C}$		275	40
PGM	Phosphoglucomutase (2.7.5.1)	I	an	275	40
SORDH	L-iditol dehydrogenase (1.1.1.14)	I	an	275	40
TPI	Triose phosphate isomerase $(5.3.1.1)$	I	an	275	40

toethanol, pH 7.0) with a tissue grinder, then centrifuged at 14,000 rpm for 8 minutes. Tissue supernatant (0.25 ul) was applied to cellulose acetate plates using the Super Z-12 application system (Helena Laboratories, Beaumont, Texas). Plates were electrophoresed in refrigerated rigs under the conditions indicated in Table 2, then stained using an agar overlay and covered to prevent back staining. Bands were scored by measuring the relative mobilities of alleles from the origin after arbitrarily assigning the most common allele a mobility value of 100. To insure consistency of scoring between runs, two individuals from each plate were run on the subsequent plate, and at least two species were always represented on a plate.

Genetic divergence between *Callosamia* species was estimated by calculating Nei's pairwise genetic identities (Nei 1978) with jackknifed standard errors across all eighteen loci (Hartl & Clark 1989). Intraspecific patterns of allele frequencies (e.g., Wright's F-statistics, overall heterozygosities) were not calculated due to the small sample sizes.

# RESULTS

Eighteen loci were resolved across the taxa surveyed. Six loci showed no variation (ALD, GPI, G6PDH, PEP-LA, SORDH, TPI), but fixed differences were observed between *Callosamia* species pairs at five loci (AC, ACP, FUM, MDH, and P3GDH), and the remaining seven loci were polymorphic in at least one species. Nine loci were polymorphic in *Hyalophora cecropia* and six were invariant; there was only one shared allele between *Callosamia* and *Hyalophora* (AAT-1). The allele frequencies and relative mobility of allozymes are summarized in Table 3.

Callosamia promethea, C. angulifera and C. securifera were distinguishable by fixed alleles at three or more loci, as expected for genetically distinct species. There were four fixed differences between C. securifera and C. angulifera (AC, ACP, FUM, MDH); and three between C. angulifera and C. promethea (AC, ACP, P3GDH). Genetic identities and jackknifed standard errors calculated from invariant, polymorphic and fixed alleles at all 18 loci indicate that C. promethea, C. angulifera and C. securifera are equally differentiated from each other with genetic identities of  $0.76\pm0.08$  (C. promethea vs. C.angulifera ),  $0.77\pm0.08$  (C. promethea vs. C. securifera) and  $0.79\pm0.08$  (C. angulifera vs. C. securifera).

## DISCUSSION

Although *Callosamia* species hybridize in captivity, their genomes are quite distinct, as evidenced by the presence of fixed allelic differences among the three taxa. The presence of fixed differences at a single locus is generally accepted as evidence of complete reproductive isolation in sympatric taxa (Menken 1989), and those between *C. promethea* and *C. angulifera* held true even in samples collected from a location where both species were abundant (Cass County, Michigan). These electrophoretic results support the generally accepted view that reproductive isolation between *Callosamia* species in the wild is complete (Ferguson 1972, Peigler 1980). The estimated genetic identities of 0.76, 0.77, and 0.79 between species pairs are within the range of values (0.32 to 0.99) reported in other studies of congeneric Lepidoptera (Stock & Castrovillo 1981, Pashley 1983, Menken 1989, Hagen & Scriber 1991) and are consistent with the view of the genus as a discrete cluster of equally differentiated species.

Although our study revealed no evidence of allelic introgression between *Callosamia* species, we cannot eliminate the possibility that low levels of introgression may occur in some geographic locations. To date, there are few or no quantitative field studies of interspecific attraction and mating frequency in *Callosamia*, and few for other saturniids (Collins & Tusks 1979). Moreover, if hybrid matings do occur, local hybrid zones may exist in parts of the geographical ranges of these moths. Because gene introgression can be assymetrical or severely reduced by post-zygotic incompatibility of hybrids or the mating behavior and ge-

TABLE 3. Allele frequencies for 18 loci resolved in *Callosamia securifera*, *C. angulifera*, *C. promethea*, hybrids and *Hyalophora cecropia*. Sample sizes (number of individuals) listed in parentheses.

		and a state of the second s					
AAT-1		(12)	(12)	(11)	(3)	(1)	(5)
	20	0.17	0.00	0.00	0.00	0.00	0.00
	70	0.12	0.04	0.00	0.00	0.00	0.00
	100	0.71	0.46	1.00	1.00	0.00	1.00
	140	0.00	0.50	0.00	0.00	0.00	0.00
	200	0.00	0.00	0.00	0.00	1.00	0.00
AAT-2		(12)	(12)	(12)	(3)	(1)	(5)
	67	0.08	0.08	0.00	0.33	0.00	0.00
	75	0.00	0.00	0.00	0.00	0.00	0.40
	100	0.92	0.92	1.00	0.67	1.00	0.00
	125	0.00	0.00	0.00	0.00	0.00	0.60
HBDH		(12)	(12)	(12)	(7)	(1)	(8)
	-250	0.00	0.00	0.00	0.00	0.00	0.13
	-200	0.00	0.00	0.00	0.00	0.00	0.75
	-100	0.00	0.00	0.00	0.00	0.00	0.13
	50	0.17	0.00	0.04	0.14	0.00	0.00
	100	0.83	1.00	0.96	0.86	1.00	0.00
IDH	57526436	(8)	(8)	(7)	(0)	(0)	(0)
	100	0.69	0.69	0.93			_
	130	0.31	0.31	0.07	_		_
LDH		(12)	(12)	(12)	(7)	(1)	(8)
	67	0.00	0.00	0.58	0.00	0.00	0.00
	100	1.00	1.00	0.42	1.00	1.00	0.00
	120	0.00	0.00	0.00	0.00	0.00	0.69
	150	0.00	0.00	0.00	0.00	0.00	0.31
MPI		(4)	(4)	(4)	(0)	(0)	(0)
	50	0.00	0.13	0.00			
	75	0.25	0.25	0.50			
	100	0.75	0.50	0.50		_	
	125	0.00	0.13	0.00			
PGM		(7)	(8)	(8)	(0)	(0)	(0)
	75	0.50	0.50	0.12			
	100	0.50	0.50	0.88			
AC		(4)	(4)	(4)	(7)	(0)	(8)
	80	0.00	0.00	0.00	0.00		1.00
	100	1.00	0.00	1.00	0.50		0.00
	150	0.00	1.00	0.00	0.50		0.00
ACP		(8)	(8)	(8)	(7)	(1)	(8)
	40	0.00	0.00	0.00	0.00	0.00	1.00
	60	0.00	1.00	0.00	0.00	1.00	0.00
	100	1.00	0.00	1.00	1.00	0.00	0.00
FUM		(12)	(12)	(12)	(7)	(1)	(8)
	62	0.00	0.00	0.00	0.00	0.00	0.56
	67	1.00	0.00	0.00	0.00	0.00	0.00
	88	0.00	0.00	0.00	0.00	0.00	0.44
	100	0.00	1.00	1.00	1.00	1.00	0.00
MDH	-	(12)	(12)	(12)	(7)	(1)	(8)
	70	0.00	0.00	0.00	0.00	0.00	0.56
	15	1.00	0.00	0.00	0.00	0.00	0.00

	90	0.00	0.00	0.00	0.00	0.00	0.44
	100	0.00	1.00	1.00	1.00	1.00	0.00
P3GDH		(12)	(12)	(12)	(7)	(1)	(8)
	30	0.00	0.00	0.00	0.00	0.00	0.50
	50	0.00	0.00	0.00	0.00	0.00	0.50
	67	0.00	0.00	1.00	0.50	0.50	0.00
	100	1.00	1.00	0.00	0.50	0.50	0.00
GPI		(8)	(8)	(8)	(7)	(1)	(8)
	80	0.00	0.00	0.00	0.00	0.00	0.38
	90	0.00	0.00	0.00	0.00	0.00	0.62
	100	1.00	1.00	1.00	1.00	1.00	0.00
G6PDH		(12)	(12)	(12)	(7)	(1)	(8)
	60	0.00	0.00	0.00	0.00	0.00	0.25
	75	0.00	0.00	0.00	0.00	0.00	0.75
	100	1.00	1.00	1.00	1.00	1.00	0.00
PEP-LA		(12)	(12)	(12)	(4)	(0)	(3)
	75	1.00	1.00	1.00	1.00		0.00
	100	0.00	0.00	0.00	0.00	_	1.00
TPI		(12)	(12)	(12)	(7)	(1)	(8)
	60	0.00	0.00	0.00	0.00	0.00	0.50
	80	0.00	0.00	0.00	0.00	0.00	0.50
	100	1.00	1.00	1.00	1.00	1.00	0.00
ALD		(12)	(12)	(12)	(0)	(0)	(0)
	100	1.00	1.00	1.00			_
SORDH		(12)	(12)	(12)	(0)	(0)	(0)
	100	1.00	1.00	1.00			

netic incompatibility of the two species (Harrison 1990), a more robust sampling protocol than the one used in our study would be required to eliminate the possibility of localized hybrid zones or very subtle degrees of genetic introgression.

Hybridization between species with distinct genetic makeups is not uncommon in other attacine saturniids, although most occur in laboratory or otherwise unnatural settings. Not only are intrageneric hybrids possible within *Callosamia*, but intergeneric hybrids have been obtained from matings of *C. promethea* X *Hyalophora cecropia* and in crosses of all three *Callosamia* species X *Samia cynthia* (Drury) (Peigler 1978, Carr 1984). However, most of these hybridizations resulted in malformed or sterile offspring, presumably due to post-mating incompatibilities such as differences in chromosome numbers (Robinson 1971). Chemical differences in mating pheromones of attacine silk moths appear to play a minor role in maintaining reproductive isolation, since there are numerous reports of cross-attraction among the three *Callosamia* species and *Hyalophora cecropia* (Rau & Rau 1929, K. S. Johnson, unpubl. data).

In our study, Hyalophora was not useful for outgroup comparison due to the low number (one) of alleles shared with Callosamia, and the equal genetic differentiation between the three Callosamia species pairs sheds little light on their intrageneric relationships. On the other hand, the electrophoretic dissimilarity between Callosamia and Hyalophora is of interest because it suggests that these taxa may not be as closely related as presumed (Michener 1952). Callosamia is currently limited to the three taxa found only in North America, but the genus may be more closely allied with other attacine lineages, such as the Asiatic Samia. Patterns of host plant use support this relationship, as both Samia and Callosamia are unique within the Attacini in their ability to utilize magnoliaceous hosts (Stone 1991). Hyalophora occasionally have been reported to feed on plants in this family (primarily tulip tree) but larval survival on it is extremely low (Scarbrough et al. 1974, Manuwoto et al. 1985). Additional studies are needed to clarify the phylogenetic relationships among Callosamia, Hyalophora, Eupackardia, and Samia.

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