Genetic Criteria for Establishing Evolutionarily Significant Units in Cryan's Buckmoth

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Abstract: *Buckmoths (*Hemileuca *spp.) are day-flying saturniid moths with diverse ecologies and host plants. Populations that feed on* Menyanthes trifoliata, *known commonly as Cryan's buckmoths, have been found in only a few bogs and fens near eastern Lake Ontario in New York and near Ottawa in Ontario, Canada. Because of their unique ecological traits, geographic isolation from other* Hemileuca *populations, and the small number of sites they occupy, there is concern that the Cryan's buckmoth populations are phylogenetically distinct and should be protected. The Cryan's buckmoths have not yet been taxonomically described and do not appear to have clear distinguishing morphological characters. Both molecular genetic traits (allozymes and mitochondrial DNA sequences) and an ecologically based character (host performance) were investigated to determine whether these populations possess fixed diagnostic characters signifying genetic differentiation from other eastern* Hemileuca *populations. Such differences would merit separate conservation management as an evolutionarily significant unit. Our studies showed that the Cryan's buckmoths clearly belong to the* Hemileuca maia *species group, but they could not be readily distinguished from other members of that group by means of molecular genetic techniques. There were no fixed differences in alleles or haplotypes distinguishing any of the populations or species, suggesting recent divergence. Nonetheless, in the host-plant performance experiment only the Cryan's buckmoth larvae were able to develop on* M. trifoliata, *a significant difference from other* Hemileuca *larvae tested. The Cryan's buckmoth appears to be unique in host performance and warrants protection and management as an evolutionarily significant unit. In cases such as this where groups appear to have recently diverged, investigations into ecologically significant traits may provide indicators of conservation significance as reliable as molecular genetic markers.*

Criterios genéticos para el establecimiento de Unidades Evolutivamente Significativas para las polillas de los pantanos

Resumen: *Las polillas de los pantanos del género* Hemileuca *spp. (*Lepidoptera: Saturniidae*) son diurnas con una variedad de plantas huéspedes y un amplio espectro ecológico. Poblaciónes de polillas que se alimentan de* Menyanthes trifoliata, *de nombre común "polillas de Cryan," han sido encontradas en pantanos de solo dos lugares: cerca de la parte este del Lago Ontario y cerca de Ottawa, Ontario, Canada. Dado a que poseen características ecológicas poco comunes, a que son geográficamente aisladas de otras poblaciónes de* Hemileuca *y a que se encuentran en pocos sitios, se cree que estas poblaciónes de polillas de Cryan son filogenéticamente distintas y deben ser protegidas. Aunque fueron descubiertas hace mas de 10 años, las Cryan polillas de todavía no han sido descritas taxonómicamente y no parecen poseer características morfológicas distintivas. Esta investigación utilizó, simultaneamente, pruebas genéticas moleculares (aloenzimas y secuencia de ADN*

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mitocondrial) y ensayos ecológicos de supervivencia en plantas específicas. El objectivo fue determinar si estas poblaciónes poseían suficiente singularidad genética en relación a otras poblaciónes similares de Hemileuca, *para poder evaluar si merecían medidas especiales de conservación como Unidad Evolutiva Significativa. Estos estudios mostraron que a polillas de Cryan evidentemente pertenecen al grupo de especies* Hemileuca maia, *pero no pudieron ser diferenciadas de otras especies del grupo a nivel molecular. No se encontraron diferencias fijas entre alelos, que hubieran permitido distinguir entre poblaciones y especies, lo que implica una divergencia reciente. Sin embargo, se detectaron diferencias significativas respecto a la supervivencia en las plantas huéspedes entre las larvas de las polillas de Cryan y las de otras especies de* Hemileuca. *Solo las larvas de Cryan lograron desarrollarse en* M. trifoliata. *Debído a que las Cryan polillas de parecen ser únicas en su capacidad de desarollo en* M. trifoliata, *se deberían tomar medidas de protección y conservación como unidad evolutiva significativa. En casos como éste, en que los grupos parecen haber divergido recientemente, puede ser que los estudios de las características de relevancia ecológica sean indicadores confiables de la importancia de la preservación, tal como lo son los métodos de genética molecular utilizando marcadores neutrales.*

Introduction

The designation of species has always been a contentious issue, but in conservation biology we often lack the time for taxonomic debate (Rojas 1992). The concept of evolutionarily significant units (ESUs) was developed as a supplemental way to describe evolutionarily distinct groups when taxonomy was either inadequate or too controversial to reflect these distinctions (Ryder 1986). The use of the ESU concept can focus attention on actual subdivisions of genetic (and evolutionary) variation. It can therefore provide a valuable tool for reaching the main objective of conservation biology, the preservation of unique ecological adaptations and the maintenance of evolutionary potential (Dizon et al. 1992; Moritz 1994; Vogler & DeSalle 1994).

There is no general agreement on the criteria that define an evolutionarily significant unit. Character-based methods have been proposed (Amato 1991; Dowling et al. 1992; Vogler & DeSalle 1994) that are grounded in the theoretical framework of the concept of phylogenetic species (Nelson & Platnick 1981; Cracraft 1983; Nixon & Wheeler 1990). According to this definition, ESUs are based on the presence of unique "diagnostic" characters that set apart groups of individuals or populations from other such groups lacking these characters. The diagnosis of individuals and populations for the identification of phylogenetic species has been formally described as population aggregation analysis (Davis & Nixon 1992). Some authors have argued that a less stringent definition would be desirable to accommodate units that are demographically subdivided but have not experienced an evolutionary divergence time sufficient to allow the accumulation of diagnostic characters (Waples 1991; Moritz 1994). The term "management unit" (MU) has recently been proposed for these less divergent populations (Moritz 1994).

Because molecular studies of populations of endangered species have become increasingly common, the assessment of ESUs has frequently been based on evidence from DNA and allozyme data. But a variety of sources, including ecological, behavioral, biogeographical, and morphological data, are equally valid in assessing conservation units. Prerequisite for the use of non-DNA data is the assumption that the observed traits are based on heritable attributes and, therefore, provide character information that is hierarchical in nature (Avise 1989; Waples 1991; Dizon et al. 1992; Vogler et al. 1993; Moritz 1994; Vogler & DeSalle 1994).

Concordance of nonmolecular and molecular data sets can provide clear evidence for identifying ESUs, but what of those cases where there is a lack of agreement? An example is Cryan's buckmoth, in which ecological differences among populations are not obviously reflected in molecular genetic data.

In the late 1970s John Cryan and Robert Dirig discovered four populations of buckmoths, *Hemileuca* sp., inhabiting a small area of sphagnum peatlands (bogs and fens) along the southeast shore of Lake Ontario in New York (J. Cryan & R. Dirig, personal communication). Later, two more populations were discovered in large fens west of Ottawa, Ontario. Extensive searches have yielded only two more small populations in New York, both clustered near a previously known site. Thus, only eight populations of this moth (Cryan's *Hemileuca*, or Cryan's buckmoth) are known, with all those in the U.S. occurring in small, sensitive wetland areas. (In 1995, too late for inclusion in this study, the occurrence of a new *Hemileuca* population was confirmed in South-eastern Wisconsin that appears to be Cryan's *Hemileuca* based on habitat and host use [J. Tuttle, personal communication].) The U.S. Fish and Wildlife Service has expressed interest in the conservation of this organism, listing it as a C2 candidate species with potential for receiving federal protection as a threatened or endangered species. But progress in determining its need for protection has been hampered because no species or subspecies description has been created for Cryan's *Hemileuca*, in

part because of the taxonomic difficulty posed by the group (J. Cryan, personal observation).

Cryan's buckmoth is morphologically extremely similar to the three other *Hemileuca* species in eastern North America, *H. maia, H. nevadensis,* and *H. lucina,* which are known collectively as the *H. maia* species group. It is found at the northeastern margin of the group's distribution (Fig. 1), and all of these organisms almost certainly share a close evolutionary history. *Hemileuca* populations from the western Great Lakes region form an apparent transition zone between species, with the ecological associations of *H. nevadensis* but with the external coloration varying such that populations from different areas match the typical wing patterns of different *maia* group species (Tuttle in press). In this region they are called *H. nevadensis/maia* intermediate, but these populations have been split up between the three species in a variety of ways (as in Ferguson 1971, Scholtens & Wagner 1994). Several *maia* group species restricted to the southwestern U.S. have been recently identified or resurrected from synonymy (Peigler & Stone 1989).

Typically, new species and subspecies are described according to unique morphological features. Cryan's buckmoth, like some other members of the *maia* group, appears to lack diagnostic morphological features that would separate it from other species in the group (Legge 1993). Instead, Cryan's *Hemileuca* has been most readily distinguished by ecological features. Most buckmoths are found in xeric habitats, especially in the southwestern U.S. and Mexico, and most of the notable ecology of Cryan's buckmoth stems from its unusual peatland habitat.

The host-plant association is unique for the *maia* group. All Cryan's buckmoth populations feed on the

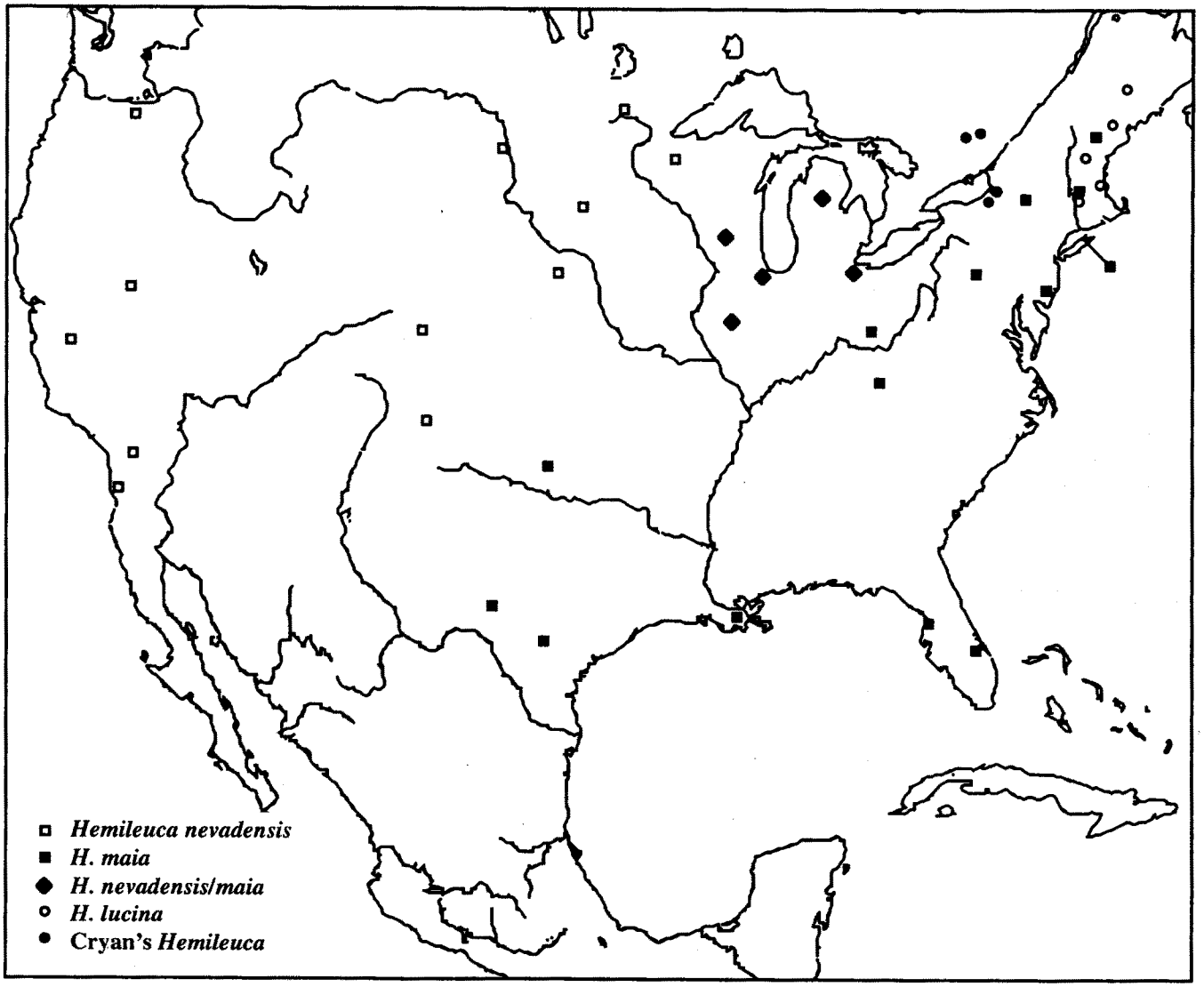

Figure 1. Locations of various extant and historic populations from the named species in the Hemileuca maia *group and Cryan's* Hemileuca *(see Table 1 and Ferguson 1971).*

wetland herb *Menyanthes trifoliata* (Menyanthaceae) and apparently nothing else, at least in their early instars. Except for a few southwestern *Hemileuca* species consuming grasses, woody rather than herbaceous hosts are the rule for the genus (Ferguson 1971; Stone & Smith 1990). Cryan's *Hemileuca* also show other adaptations to life in the peatlands. Buckmoths overwinter as eggs laid in clusters called eggrings. Normally these are laid around a woody stem of the host, but *M. trifoliata* is an herb whose leafstalks die off with the frost and sprout each spring from a submerged rhizome. Thus, unlike those of other known *Hemileuca*, the eggrings of Cryan's buckmoth are laid around woody stems of other nearby non-host plants from which larvae must crawl to find the host. Most buckmoth species burrow several centimeters underground to pupate, but the waterlogged sphagnum beds where Cryan's *Hemileuca* live limit this behavior. Captive-reared Cryan's *Hemileuca* larvae pupate much closer to the surface than those of most species in the genus. Under natural conditions larvae presumably burrow into some of the higher hummocks to find a sheltered spot dry enough for pupation (J. Cryan, personal observation).

Host-plant relationships in the rest of the *maia* group have proven to be ambiguous and confusing in many studies (Leeuw 1974; Smith 1974; Stamp & Bowers 1986; Cryan & Dirig 1987; Metzler & Lucas 1990; Tuttle in press). This status has led some entomologists to suggest that the group constitutes one widespread species exhibiting a great deal of regional variation (Scholtens & Wagner 1994). Under that hypothesis the Cryan's *Hemileuca* would be only a particularly interesting regional variant within this complex of clines. Traditionally, though, systematists have recognized the three *maia* species previously mentioned and have noted that their separation is far from clear-cut, particularly for *H. maia* and *H. nevadensis* (Ferguson 1971; Peigler & Stone 1989).

Our objective was to determine whether diagnostic characters exist to separate the Cryan's *Hemileuca* from similar eastern *Hemileuca* species, using both molecular genetic and ecological traits. We studied allozyme variation, mitochondrial DNA (mtDNA) sequence variation, and host-plant performance in the laboratory. We compared Cryan's *Hemileuca* to members of the *H. maia* species group (the only other *Hemileuca* in eastern North America): *H. lucina, H. maia, H. nevadensis,* and the *H. nevadensis/maia* intermediate.

Methods

Allozymes

Allozyme electrophoresis was carried out on representatives of 16 populations from the *H. maia* group collected from across North America (Table 1). Most specimens for electrophoresis were collected alive as eggs or larvae and reared on their natural host until adulthood. Some adults were collected in the field. All specimens were frozen alive at −80°C in the fall of 1991 (except for *H. oliviae*, which were collected and frozen in 1989 by J. DuBach).

Two populations of Cryan's *Hemileuca* were included, one each from New York and Ontario. Other sampled populations from the *H. maia* group included one population of *H. lucina*, four of *H. maia*, three of *H. nevadensis*, and two of the *H. nevadensis/maia* intermediate. Populations were selected to represent the geographic spectrum of their species, including some populations located as close as possible to the range of Cryan's *Hemileuca*. One to five individuals from each population were analyzed. For outgroups we used specimens from populations of *Hemileuca hera, H. eglanterina* (both of the subgenus *Pseudohazis*), and *H. oliviae* (subgenus *Euleucophaeus*). The *maia* group belongs to

Table 1. Populations used in studies of interpopulation variation in *Hemileuca*.

Subgenus	*Species*	*Abbreviation*	*Population origin*
Hemileuca	uncertain (Cryan's buckmoth)	Cbm-bb	Northern Oswego Co., New York
		Cbm-mp	Southern Oswego Co., New York
		Cbm-rf	Richmond Fen, Ontario
Hemileuca	*lucina*	HlMA	Franklin Co., Massachusetts
Hemileuca	*maia*	HmMA	Franklin Co., Massachusetts
		HmNY	Suffolk Co., New York
		HmNJ	Ocean Co., New Jersey
		HmLA	Baton Rouge Parish, Louisiana
Hemileuca	*nevadensis/maia*	Hn/mOH	Lucas Co., Ohio
		Hn/mMI	Roscommon Co., Michigan
Hemileuca	*nevadensis*	HnWI	Douglas Co., Wisconsin
		HnCO	Arapahoe Co., Colorado
		HnCA	Merced Co., California
		HnNM	Otero Co., New Mexico
Pseudohazis	*eglanterina*	H(P)e	Riverside Co., California
Pseudohazis	*hera*	H(P)h	Douglas Co., Nevada
Eulencophaeus	*divia*	H(E)o	Trinidad Co., New Mexico

the subgenus *Hemileuca*, so its members are presumably not as closely related to the outgroup species.

Electrophoretic procedures followed those of May (1992). Whole, individual moths with wings removed were crushed and ground in a solution of 0.05 mol/dm^3 Tris-HCl buffer (pH 7.1). No siblings were included in the analysis. Samples were taken from these homogenates and exposed to horizontal starch-gel electrophoresis coupled with histochemical staining. Remaining sample materials were stored at −80°C. Virtually all adults used were male; the few females had their abdomens removed to prevent any complications from paternal contributions to their eggs. The most common allele was designated as "1" for each locus, with subsequent alleles designated as 2, 3, and so forth. The normalized genetic distances (*D*) between the populations were calculated by Nei's methods (1978), which include a correction for sampling error. Allele counts by locus between and within appropriate population groupings were compared statistically by contingency-table analysis with *G*-tests (Sokal & Rohlf 1981). Statistics were calculated with the program "Genes in Populations Two" (designed by B. May and C.C. Krueger and written in C by W. Eng and E. Paul).

Mitochondrial DNA Sequencing

The 16 populations used for sequencing included most of those used for the allozyme analysis. One more of the New York populations of Cryan's *Hemileuca* was included, whereas the outgroup species *H. bera* was not available for use.

Total genomic DNA was extracted from a single individual from each population using the protocol of Vogler and DeSalle (1993). Primers used for PCR amplification were designed to amplify a fragment corresponding to positions 3340 to 3499 of the published *Drosophila yakuba* sequence in the cytochrome oxidase II gene (Clary & Wolstenholme 1985). The primary PCR product of 750 bp was cloned into a multicopy plasmid using a commercial cloning kit (TA cloning; Invitrogen, La Jolla, Calif.) and partially sequenced with a manufactured kit (Sequenase, United States Biochemicals). Internal primer sequences were designed from regions that had high correlation with the *Drosophila yakuba* sequence. These primers (5'ATTTGAACAATTTTACCTGC and 5'CTGAGGCAGTAATTAGAATACGAATTTG) were used to amplify and sequence all of the *Hemileuca* mtDNA isolates.

The published CO-II sequence of *Galleria mellonella* (Pyralidae, a more ancestral lepidopteran family than Saturniidae) was used as a basal outgroup (Liu & Beckenbach 1992). The CO-II gene has been used to resolve taxa in *Apis* (Apidae: Hymenoptera; Garnery et al. 1991), where 270 bp of sequence from CO-II showed patterns of relationship among the species and subspecies.

The phylogenetic analysis was accomplished with PAUP 3.0s (Swofford 1990). The pyralid *G. mellonella* was used to root the tree.

Larval Host-Plant Performance

The host-feeding ability of newly emerged larvae from one New York population of Cryan's *Hemileuca* was compared with those of geographically close populations of *H. maia* and the *H. nevadensis/maia* intermediate. All were collected from the field in 1992 as eggs. Sampled populations included a Cryan's *Hemileuca* population near Pulaski, Oswego County, New York (the largest population in New York); *H. nevadensis/maia* intermediate from Lucas County, Ohio; and *H. maia* from near Riverhead, Suffolk County (Long Island), New York.

H. maia consumes a variety of species of *Quercus*, especially *Q. ilicifolia* in the Northeast, whereas *H. nevadensis* consumes many species of *Salix*. Different host-use patterns are often observed between conspecific, allopatric population of insects and may often be involved in speciation or adaptive radiation (Thompson 1988; Hagen 1990), at least partially because of the greater efficiency of food utilization through specific ecological or metabolic adaptation to different hosts (Scriber 1983). Among the host plants of *Hemileuca* we examined, clear differences in predominant secondary compounds existed that could influence host specialization (Tiitto 1986; Scalbert & Haslam 1987; Bowers 1988; Faeth 1992). Previous work has shown that *H. maia* grows well on *Quercus rubra* and that *H. nevadensis* grows readily on *Salix nigra* (unpublished observations). In a greenhouse *Q. rubra* seedlings were grown from acorns, and *S. nigra* cuttings were rooted and induced to sprout new leaves. We purchased rhizomes of *Menyanthes trifoliata* for Cryan's *Hemileuca* from an aquatic garden-supply company, which were also planted and grown in the greenhouse.

Eggrings, kept cool following collection to simulate winter conditions, were placed in the greenhouse kept at 24°C. Larvae developed and eclosed in about three weeks. Almost all of the larvae from a given eggring hatched over the span of a few hours. Newly emerged larvae were first divided into as many sibling groups of 14 to 17 individuals (usually 15) as possible. Larvae were kept in groups of this size because of their gregarious nature in the early instars, which makes them difficult to rear alone or in smaller groups.

Each sibling group was assigned to one of the three host plants, with about one-third of the total number of groups from each eggring on each host. A few eggrings were so small that only one sibling group per host was possible, but most eggrings provided three or four groups per host. At least one sibling group from every eggring was assigned to each of the three hosts. The insects were placed on a leaf of the host plant, and a mesh

sleeve was placed over the larvae along the stem, with foam plugs on the ends to seal the bag to the stem. Larvae were checked at 3-day intervals over 12 days. The presence of dead larvae was recorded, and starvation was assumed to be the cause of all deaths. At the final check all larvae were accounted for as either living, dead, or escaped.

The statistical significance of larval population origin on performance was tested for each host by comparing the mean proportion of larval survival for each population through 12 days. The mean survivorship for each population on each host was obtained by taking the means of the survivorship rate for all of the individual larval sibling groups from each eggring that were placed on a given host and then producing an overall mean for that host using the means from all of the eggrings of a population. These data were analyzed using the Kruskal-Wallis test (Lehman 1975) to compare the performance of the populations on each of the three host plants. This analysis uses a contingency table to determine whether the exact distributions of the survivorship values for each population differ. For each table, survivorship data was divided into four categories: 0–0.24, 0.25–0.49, 0.50–0.74, and 0.75–1.0. Thus, three contingency tables were produced, one discerning the significance of feeding differences for each of the three hosts.

Results

Allozymes

In allozyme electrophoresis of adult *Hemileuca* the products of 15 loci were resolved, with 13 polymorphic (glucose-6-phosphate isomerase [Gpi], malate dehydrogenase-1 and -2 [Mdh-1, Mdh-2], glutathione reductase [Gr], aspartate amino transferase-1 and -2 [Aat-1, Aat-2], peptidase [with glycyl-leucine] [PepGL], malic enzyme [Me], triosephosphate isomerase [Tpi], isocitrate dehydrogenase [Idh], lactate dehydrogenase [Ldh], adenylate kinase-1 [Ak1], and superoxide dismutase [Sod] (Appendix 1). Only one of these (PepGL) showed significant polymorphism within the *maia* group (including Cryan's *Hemileuca*), but it did not provide a diagnostic character differentiating any taxa. The remaining loci showed polymorphism largely in the outgroups (*H. hera, eglanterina,* and *oliviae*) or between them and the *maia* group.

Genetic distances (Nei 1978) based on all loci and corrected for sample size show that all of the *maia* group populations are relatively similar, with a maximum value of 0.230 between the most distant populations (Wisconsin *H. nevadensis* and New York *H. maia*). A dendrogram of genetic distance clustered by UPGMA links *maia* group populations in a pattern that is incoherent according to geography, ecology, or currently defined taxa (Fig. 2). Most of the populations demonstrate very low distances when compared to one another, and all are well separated from the three outgroups. Fixed differences separating outgroups from the *maia* group existed at 7 loci for *H. oliviae*, 5 loci for *H. hera*, and 4 loci for *H. eglanterina*. In comparing allele-frequency homogeneity using the summed *G*-test over all loci, significant differences were found only when individual or pooled *maia* group populations were tested against the outgroups (Table 2). No differences significant at $p < 0.05$ were found in within-*maia* group comparisons, either with all populations considered individually, polled to compare Cryan's *Hemileuca* to all other *maia* group populations, or in pairwise comparisons (not shown).

It is possible that the genetic distances between *maia* group populations could be resolved by using larger sample sizes, and that this could potentially reveal the relationships among them through allozyme frequency differences. But, for two reasons, we focused instead on finding distinct, diagnosable characters. First, there are only a handful of Cryan's buckmoth populations, and most of them undergo drastic fluctuations in numbers of individuals from one season to the next, with very few adults in some years (J. Cryan, personal observation). By limiting ourselves to methods requiring smaller sample sizes, we expended the least amount of our most critical resource, the rare organism itself. Second, basing an evolutionarily significant unit of Cryan's *Hemileuca* on anything less definitive than a diagnostic character would

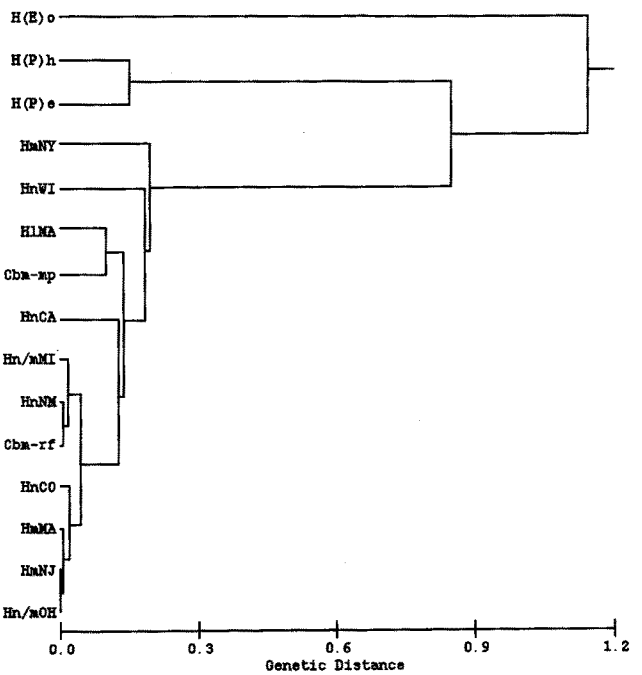

Figure 2. Dendrogram showing relationships of Hemileuca *populations based on Nei's genetic distances calculated from allozyme variation at 15 resolvable loci. Abbreviations are defined in Table 1.*

Table 2. Average F_{st} and G-test statistics summed over 13 polymorphic allozyme loci observed among samples of Cryan's *Hemileuca*, other *H. maia* group populations, and more distantly related *Hemileuca* species.

Source	Average F_{st}, all loci (range)	Total		Number of loci with a significant G-value at	
		G	df	$p < 0.05$	$p < 0.01$
All samples	0.851 (0.302-1.000)	1006	434	10	10
Pooled *H. maia* group populations versus outgroups	0.898 (0.074-1.000)	928	93	12	12
Among all *H. maia* group populations	0.389 (−0.161-0.478)	78	88	0	0
Pooled Cryan's *Hemileuca* populations versus all other *H. maia* group populations pooled	0.087 (0.000-0.106)	9.755	8	0	0

leave the ESU too open to question and revision, because a lack of clarity already exists in the taxonomic distinction of *maia* group species currently described. Therefore, enlarging the sample sizes for allozyme sampling would have been of limited value and could have had a detrimental effect on populations of Cryan's *Hemileuca*. Had we discovered potentially fixed differences separating Cryan's buckmoth in our original allozyme analysis, we would necessarily have increased the sample size to confirm the diagnosability of the characters.

mtDNA

We were able to obtain 160 bp of sequence from all but one of the *Hemileuca* specimens. Seven different haplotypes were found within the genus *Hemileuca* and are identified in Fig. 3. *H. (Psuedohazis) eglanterina* and *H. (Euleucophaeus) oliviae* each had their own haplotypes, which were quite different from all others and were designated as II and III respectively. A single haplotype, designated haplotype I, occurred in representatives of 12 of the 15 *H. maia* group populations (including Cryan's *Hemileuca*). The remaining three populations (*H. nevadensis* from New Mexico and California and *H. maia* from Louisiana) and one of the *H. maia* from New Jersey each had haplotypes that differed slightly (only 1-3 nucleotides changes) from type I and from one another. These types were designated Ia through Id.

Fifty of the 160 nucleotides were polymorphic overall, with 41 sites polymorphic within the genus *Hemileuca*. Within the *maia* group populations, however, only five nucleotide sites contained polymorphisms (Fig. 3), at positions 46, 103, 111, 124, and 135.

An exhaustive search was performed on the haplotypes using PAUP 3.0, combining all individuals with identical sequences. The haplotype found in the pyralid moth *Galleria mellonella* (Liu & Beckenbach 1992) was used as the outgroup. Five most parsimonious trees were produced, each with a length of 80. A strict consensus tree for these five trees united all 14 populations of the *H. maia* subgenus complex, a polytomy in which all of the groups come off the tree at the same point (Fig. 4) without resolving any relationships between them.

The recognition of diagnosable populations can be confounded by a number of sampling errors, such as the undersampling of either the number of populations, the number of individuals per population, or the number of characters sampled per individual (Davis & Nixon 1992). We analyzed only one individual from each population of *Hemileuca* in the mtDNA sequencing, and this analysis may make it impossible to recognize characters diagnostic for all individuals in a population. But the populations of Cryan's *Hemileuca* under consideration for ESU status could not be differentiated from the predominant mtDNA haplotype found in most populations of other ecological forms of the *maia* group throughout much of North America. As with the allozyme data, the inclusion of additional individuals from populations of Cryan's *Hemileuca* and other populations cannot result in the discovery of characters that are diagnostic for Cryan's *Hemileuca* populations only. Therefore, it was not meaningful to add more individuals for an evaluation of ESU status using the criterion of diagnosability.

Host-Plant Performance

In contrast to the molecular genetic studies, the host performance experiment showed dramatic differences between a population of Cryan's *Hemileuca* and representative populations of *H. maia* and *H. nevadensis/maia* intermediate in the performance of newly closed larvae on their three respective hosts (Table 3). The Cryan's *Hemileuca* larvae were the only ones to readily consume and gain weight on *Menyanthes trifoliata*, their natural host, with 100% survivorship. Larvae from the other two populations all starved to death when offered only *M. trifoliata*, and they produced no apparent feeding damage. Differences between the three main populations were highly significant on all three hosts ($p < 0.0001$). In some cases standard error was zero because all larvae in all test groups of one population on a partic-

Haplotype	Population	1					60
I	Many*	ACTAGACGAA	TTAAATAACC	CCTTACTTAC	ATTAAAATCA	ATTGGCCACC	AATGATACTG
I	HmNY				-------	----------	----------
Ia	HmNJ-1	----------	----------	----------	----------	----------	----------
Ib	HmLA	----------	----------	----------	----------	----------	----------
Ic	HnCA	----------	----------	----------	----------	----------	----------.
Id	Ha	----------	----------	----------	----------	-----T----	----------
II	H(P)e	-T----T---	C----C--T-	--C--A----	C--------T	-----T--T-	-------T--
III	H(E)o	-T----T---	-----C----	-T---A----	T---------	-----G--T-	----------
out	Gm	-T----T---	--------T-	-TC-TA----	C------A-T	-----A--T-	-------T--

Haplotype	Population	61					120
I	Many*	AAGTTATGAA	TATTCTGATT	TTAAAAATAT	TGAATTTGAT	GCTTATATAA	TCCCAACTAA
I	HmNY	----------	----------	----------	----------	----------	----------
Ia	HmNJ-1	----------	----------	----------	----------	----------	C---------
Ib	HmLA	----------	----------	----------	----------	----------	----------
Ic	HnCA	----------	----------	----------	----------	--C-------	C---------
Id	Ha	----------	----------	----------	----------	----------	C---------
II	H(P)e	---A------	--C-------	-C--------	----------	-----C----	C---TT-A-C
III	H(E)o	----------	-----A--C-	----------	---------C	T-A-------	-T---T-A--
out	Gm	----------	-----A--C-	----T-----	---------C	T---------	-TG---G---

Haplotype	Population	121			160
I	Many*	TGAGTTAACC	CCTAGAAATT	TTCGTCTTTT	AGATGTAGAC
I	HmNY	----------	----------	----------	----------
Ia	HmNJ-1	----------	----------	----------	----------
Ib	HmLA	----------	----A-----	----------	----------
Ic	HnCA	---A------	----------	----------	----------
Id	Ha	----------	----------	----------	----------
II	H(P)e	---A------	---GA-----	-----T-AC-	----------
III	H(E)o	---AC-T-AT	A---C---C-	-----T-A--	---------G
out	Gm	---AC-TC-T	TTA-AT----	----------	---------T

Figure 3. The 160 bp nucleotide sequence from the middle of the mitochondrial CO-II gene, between primers N-1 and N-2. Haplotypes are indicated by the Roman numerals at the beginning of each line. The "many" population includes Cbm-bb, Cbm-mp, Cbm-rf, HI, HmMA, HmNJ-2, HnOH, HnMI, HnWI, and HnCO. Abbreviations for Hemileuca *populations are defined in Table 1; "Gm" signifies the outgroup* Galleria mellonella. *Dashes signify a nucleotide identical to that found in haplotype I; spaces are left for nucleotides that could not be read. Bold signifies sites that contain polymorphisms within the* maia *group populations.*

ular host either survived or starved to death during the 12-day trial.

On *Quercus rubra*, the *H. maia* of Long Island and the *H. nevadensis/maia* intermediate of Ohio showed a significant ability to consume and gain weight. They had similar survivorships of 0.62 and 0.57 respectively, but with large standard errors. The error reflected a wide variation in performance both between different eggrings and between replicate trials of larvae from the same eggring. The Ohio population naturally consumes *Salix*, but the habitat site does contain *Quercus*. But the Long Island larvae would normally consume *Quercus ilicifolia*, so their variable performance on this similar oak was surprising. It may reflect some unknown difference between the host species. The Cryan's *Hemileuca* population showed little ability to use *Q. rubra*, with only two individuals feeding on the host and surviving the 12 days of testing.

Cryan's *Hemileuca* and the Ohio *nevadensis/maia* intermediate shared similarly high survival on *Salix nigra*. The Ohio population naturally consumes species of native willows. Cryan's *Hemileuca* larvae showed 100% survival, equal to their performance on *M. trifoliata* and highest of any population, even though they are not known to consume this host in the wild. Long Island *H. maia* larvae showed no ability to use the plant and uniformly starved to death.

Discussion

The molecular investigations indicate that the differentiation between these *Hemileuca maia* group populations is quite low, providing no diagnostic characters to separate molecularly based ESUs within the entire species group. But large differences were seen in the eco-

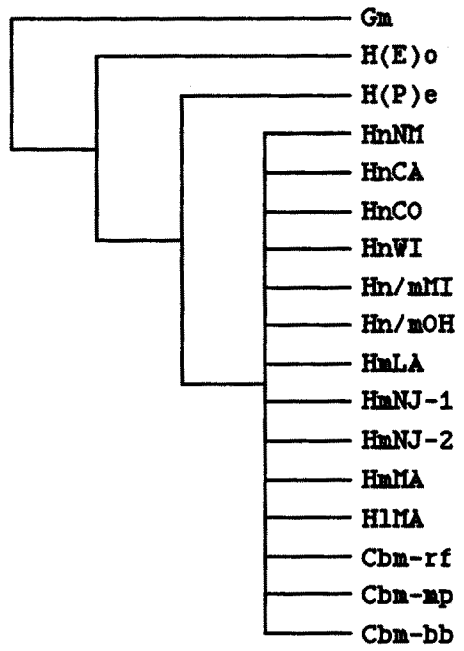

Figure 4. Strict consensus tree for Hemileuca *populations based on 160 bp nucleotide sequence from the mitochondrial CO-II gene. "Gm" denotes the outgroup* Galleria mellonella. *The other abbreviations are defined in Table 1.*

logically significant trait of first instar larval host plant use.

In the allozyme data set some populations exhibit alleles not seen in others (e.g., *H. nevadensis* from Colorado and California and *H. maia* from Massachusetts), but there are no fixed differences between any *maia* group populations at any of the loci examined (Appendix 1). Because few individuals from each population were analyzed for allozymes, it is probable that their populations actually contain more alleles (perhaps at low frequencies) than were seen here (Hillis 1987). Depending upon the time since isolation, however, reproductively isolated species or populations would be expected to have some readily observable fixed allelic differences. The large differences seen between the *Hemileuca* outgroups and the *maia* group populations are much more typical for congeneric species. Even *H. hera* and *H. eglanterina*, sympatrically occurring species in the subgenus *Pseudohazis*, show some allelic differences (at the loci Gpi and Ak-1). No such differences are evident between any of the *maia* group species.

However, *H. hera* and *H. eglanterina* do have a relatively low genetic distance for conspecific species (Thorpe 1982), at 0.122. Though all the distance values reported here should be considered tentative because of the small sample sizes, it is notable that this value is within the range of the distances found among *maia* group populations. *H. hera* and *H. eglanterina* are sympatric species that are reproductively isolated via different sex pheromones (Collins & Tuskes 1979). These pheromone data and the results of our research may provide evidence that some species within *Hemileuca* are of recent origin, with low overall molecular divergence.

Situations similar to that within the *maia* group have been described in other allozyme studies of Lepidoptera, in which groups that were ecologically or morphologically distinguishable lacked fixed allelic differences. Britten and Brussard (1990) found that the endangered nymphalid *Boloria acrocnema* actually had a very low genetic distance from allopatric populations of *B. improba*, between 0.127 and 0.134. They suggest that *B. acrocnema* may not yet have fully differentiated to the species level. In the Nepticulidae most species appear to be separated by extremely large genetic distances. But Menken (1990) found three distinct, sympatric species of nepticulids that were indistinguishable allozymatically, with distance values less than 0.051. He proposed that their allelic distribution patterns suggest recent speciation without genetic bottlenecks.

As genetic material transmitted uniparentally, mtDNA genes generally exhibit a shorter time both for fixation and loss of neutral alleles than does nuclear DNA (Birky et al. 1983). Therefore, populations tend to show

Table 3. Mean survivorship for larval sibling groups from three populations of *Hemileuca* during the first 12 days after hatching.*

Population origin	*Natural host*	*Eggrings tested*	*Survivorship (standard error)/no. sibling groups/ total no. larvae*		
			on Menyanthes trifoliata	*on* Quercus rubra	*on* Salix nigra
Cryan's buckmoth Oswego Co., NY	*Menyanthes trifoliata*	8	1.0 (0) 19 sib. groups 292 larvae	0.01 (0.01) 22 sib. groups 345 larvae	1.0 (0.1) 20 sib. groups 309 larvae
H. maia Suffolk Co., NY	*Quercus ilicifolia*	7	0.0 (0) 13 sib. groups 190 larvae	0.62 (0.28) 20 sib. groups 286 larvae	0.0 (0) 17 sib. groups 236 larvae
H. nevadensis/maia intermediate Lucas Co., OH	*Salix* sp.	6	0.0 (0) 21 sib. groups 308 larvae	0.57 (0.31) 24 sib. groups 355 larvae	0.85 (0.19) 22 sib. groups 322 larvae

**Groups were provided with one of three host plants as a food source:* Menyanthes trifoliata, Quercus rubra, *or* Salix nigra.

greater subdivision when mtDNA is examined, as well as greater loss of variability during bottlenecks (Harrison 1991). Studies of population subdivision in both *Daphnia* and *Drosophila* have found greater differences with mtDNA than with allozymes (DeSalle et al. 1987; Crease et al. 1990). This was not the case with the *Hemileuca maia* group, where no populations or species were distinguishable.

Our focus on diagnosable characters takes the most conservative and least resource-expendable approach possible. By first examining single individuals from populations we established whether the population aggregation analysis (Davis & Nixon 1992) could diagnose any of the populations or groups with minimal damage to the rare populations. If any one of the individuals in our sample had a divergent mtDNA sequence or allozyme profile, we would have been forced to examine several more individuals from each population. Given that all individuals from the various populations we examined had virtually the same mtDNA haplotype and allozyme profile, there is no possible way to extract a diagnosable character for these populations; hence, we rely on the ecological character for diagnosis.

Frequency-dependent approaches for the evaluation of conservation units (Waples 1991; Moritz 1994) rely on the analysis of larger numbers of individuals. The evaluation of conservation priorities based on these methodologies depends on a more subjective assessment of what amount of difference in allele frequency is significant to justify the establishment of conservation units. Our limited survey of allozyme data does not indicate significant differences in allele frequency of Cryan's *Hemileuca* populations, and the survey of mtDNA would have to be expanded to include additional DNA segments to detect any marker variation at all. Therefore, if criteria other than diagnosability are used to evaluate the observed variation in genetic markers, our conclusions would not be affected. The acceptance of the diagnosability criterion in our study circumvents taxonomic questions and provides a conservative estimate of the number of evolutionary lineages in the *H. maia* complex.

In sharp contrast to both mtDNA and allozymes, the data collected on host performance supports the hypothesis that Cryan's *Hemileuca* is differentiated from populations of *H. nevadensis* and *H. maia*. The larvae appear to be unique in their ability to consume and grow on *Menyanthes trifoliata*, for which the other populations showed no capability. Thus, the unique habitat and host use of this organism suggest that these populations are evolutionarily divergent in ecologically significant ways.

It is probable that all of Cryan's *Hemileuca* populations have similar host-plant feeding abilities, based on their apparently exclusive use of *M. trifoliata* in the field. Because we were able to test only one population, further trials confirming these results with remaining populations (and other *maia* group populations) should be completed before Cryan's *Hemileuca* can be defined as a monophyletic unit. We strongly suspect that it possesses monophyly.

Besides managing genetic diversity, it is a conservation goal to preserve specific adaptive parameters that populations may have accumulated in response to biotic and abiotic factors in their environments (Waples 1991; Vogler & DeSalle 1994). Molecular studies on presumably neutral markers such as mtDNA are frequently used as a proxy to evaluate the possibility of ecological differentiation (Dizon et al. 1992). In the case of Cryan's *Hemileuca*, direct evidence for ecological separation is available and can be used to recognize evolutionarily significant differentiation. It is therefore a conservative approach to manage populations of Cryan's buckmoth as a separate ESU even in the absence of supporting evidence from molecular data.

Whereas it is desirable to accumulate different types of evidence for the evaluation of ESU status, corroborating information is not always easily found, in particular during evolutionarily early stages of differentiation. There are numerous studies of endangered species and groups of conservation interest in which patterns of mtDNA variation do not precisely reflect the variation in other parameters. Examples include the species flocks of East African cichlid fishes in which substantial morphological and ecological variation occurred in the absence of genetic differentiation of mtDNA (Meyer et al. 1990), and red wolf (*Canis rufus*) populations that can be distinguished from related taxa based on morphological characters, also without any detectable mtDNA differences (Wayne & Jenks 1991). Similar issues have been considered in the conservation of endangered stocks of anadromous Pacific salmon, in which unique, reproductively isolated groups exist without quantifiable genetic differences (Behnke 1993).

In other cases, no clear morphological or ecological variation was observed despite substantial mtDNA divergence, such as in the Atlantic and Gulf of Mexico populations of endangered seaside sparrows and several unrelated taxa with similar geographic distributions (Avise & Nelson 1989, Avise 1992; see also references in Moritz 1994).

The large differences between the ecological and the molecular genetic data sets may at first seem surprising, yet they are consistent with a hypothesis of recent evolutionary divergence of the *maia* group. This species group is unusual within its genus, in which it is the most distant from the genus's center of diversity and of origin in the southwestern U.S. and northern Mexico. *H. maia* is the only member of the group found in a xeric habitat, which is typical for *Hemileuca*. The mesic and wetland habitats of *H. nevadensis, H. lucina,* and Cryan's *Hemileuca* are dramatically out of character in their genus. As

originally suggested by J. Cryan (personal communication), the various species in the group may be the result of divergence that took place in different Pleistocene refugia. The lack of clear-cut morphological differences and the species group's ecological and geographic differences from most of the genus provide some evidence for this. The lack of distinct molecular genetic variation we found across the species group is another intriguing piece of evidence.

We agree with Erwin (1991) that in conservation efforts we need to remember units like the *maia* group that appear to be "evolutionary fronts"—groups in which recent speciation has occurred and that may therefore hold the future potential of maximum biodiversity.

Although examination of genetic variation through molecular techniques can provide detailed evolutionary data, it rarely if ever directly reveals the genetic basis of ecologically significant traits. We therefore conclude that, at least for recently evolved taxa, ecological traits such as host-plant use can provide a guide to the identification of evolutionarily significant units equally reliable as some of the popular molecular genetic techniques.

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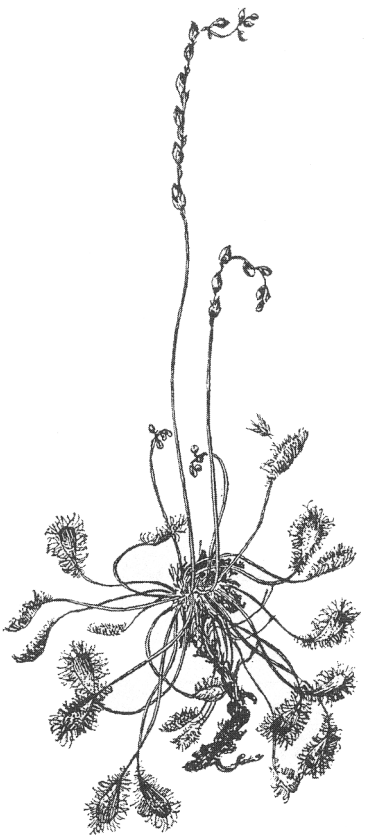

Appendix 1. Allele frequencies for the 13 polymorphic loci found in populations of *Hemileuca* species analyzed as adults.

						Hn/m						*Cbm-*				
Locus	*Allele*	*HnCA* (*n* = 3)	*HnCO* (*n* = 3)	*HnWI* (*n* = 2)	*HnNM* (*n* = 2)	*MI* (*n* = 1)	*OH* (*n* = 2)	*HmNY* (*n* = 3)	*HmNJ* (*n* = 3)	*HmMA* (*n* = 3)	*HlMA* (*n* = 2)	*rf* (*n* = 5)	*mp* (*n* = 1)	*H(P)b* (*n* = 3)	*H(P)e* (*n* = 3)	*H(E)o* (*n* = 3)
Gpi	1	1.00	1.00	1.00	1.00	0.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—	—	—
	2	—	—	—	—	0.50	—	—	—	—	—	—	—	0.17	1.00	—
	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.00
	4	—	—	—	—	—	—	—	—	—	—	—	—	0.50	—	—
	5	—	—	—	—	—	—	—	—	—	—	—	—	0.33	—	—
Mdh-1	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—	—	1.00
	2	—	—	—	—	—	—	—	—	—	—	—	—	1.00	1.00	—
Gr	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—	—	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—	—	—	—	—	1.00	1.00	—
	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.00
Aat-1	1	0.83	0.83	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	1.00	—
	2	0.17	0.17	—	—	—	—	—	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—	—	—	—	—	0.17	—	—
	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.00
Aat-2	1	*	1.00	1.0*	1.00	1.00	1.00	*	1.00	1.00	1.0*	1.00	1.00	1.00	1.00	—
	2	*	—	—*	—	—	—	*	—	—	—*	—	—	—	—	—
	3	*	—	—*	—	—	—	*	—	—	—*	—	—	—	—	0.17
	4	*	—	—*	—	—	—	*	—	—	—*	—	—	—	—	0.83
PepGL	1	0.67	—	0.50	1.00	1.00	0.25	0.33	0.33	—	0.75	0.70	1.00	—	—	—
	2	0.33	1.00	0.50	—	—	0.75	0.67	0.67	0.83	0.25	0.30	—	0.83	1.00	—
	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	4	—	—	—	—	—	—	—	—	0.17	—	—	—	—	—	—
	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.00
	6	—	—	—	—	—	—	—	—	—	—	—	—	0.17	—	—
Tpi	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	0.33	—
	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.00
Me	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—	—	1.00
	2	—	—	—	—	—	—	—	—	—	—	—	—	1.00	1.00	—
Idh	1	0.67	0.50	*	1.00	1.00	1.00	1.00	1.00	0.83	1.0*	1.00	1.00	1.00	1.00	1.00
	2	0.33	0.50	*	—	—	—	—	—	—	—*	—	—	—	—	—
	3	—	—	—	—	—	—	—	—	0.17	—*	—	—	—	—	—
Ak-1	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—	1.00	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	1.00	—	1.00

Appendix 1. Continued

						Hn/m						*Cbm-*				
Locus	*Allele*	*HnCA (n = 3)*	*HnCO (n = 3)*	*HnWI (n = 2)*	*HnNM (n = 2)*	*MI (n = 1)*	*OH (n = 2)*	*HmNY (n = 3)*	*HmNJ (n = 3)*	*HmMA (n = 3)*	*HlMA (n = 3)*	*rf (n = 5)*	*mp (n = 1)*	*H(P)b (n = 3)*	*H(P)e (n = 3)*	*H(E)o (n = 3)*
Sod	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—	—	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	1.00	1.00	—
	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.00
Ldh	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—	—	—
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.00
	3	—	—	—	—	—	—	—	—	—	—	—	—	1.00	1.00	—
Mdh-2	1	0.75*	1.00	*	1.00	1.00	1.00	*	1.00	1.00	*	1.00	*	—	—	—
	2	0.25*	—	*	—	—	—	*	—	—	*	—	*	1.00	1.00	—
	3	—*	—	*	—	—	—	*	—	—	*	—	*	—	—	1.00

**One or all of the individuals from the marked population produced poorly resolved, unreadable marks on the gel for that locus.*