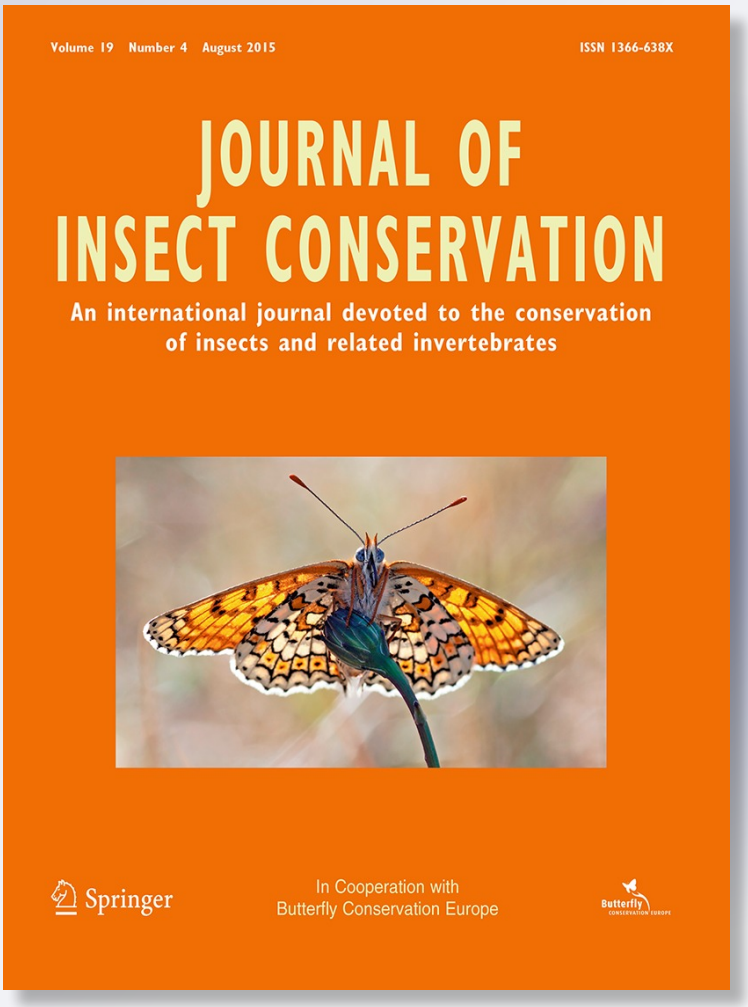
Is the globally rare frosted elfin butterfly (Lycaenidae) two genetically distinct host plant races in Maryland? DNA evidence from cast larval skins provides an answer

Jennifer A. Frye & Robert K. Robbins

Journal of Insect Conservation
An international journal devoted to the conservation of insects and related invertebrates

ISSN 1366-638X
Volume 19
Number 4

J Insect Conserv (2015) 19:607-615
DOI 10.1007/s10841-015-9783-4



Springer

J Insect Conserv (2015) 19:607–615
DOI 10.1007/s10841-015-9783-4

ORIGINAL PAPER

Is the globally rare frosted elfin butterfly (Lycaenidae) two genetically distinct host plant races in Maryland? DNA evidence from cast larval skins provides an answer

Jennifer A. Frye[1] · **Robert K. Robbins**[2]

Received: 12 January 2015 / Accepted: 4 June 2015 / Published online: 23 June 2015

Abstract Frosted elfin butterfly caterpillars (*Callophrys irus*) eat either lupine (*Lupinus perennis*) or wild indigo (*Baptisia tinctoria*) legumes. Data from larval behavior, adult morphology, demographics, and phenology have led to the suggestion that lupine-feeding populations are genetically distinct from wild indigo-feeding populations. Frosted elfins are of conservation concern throughout their range in the eastern half of North America, and the possibility of host plant races—in which females pass genetically determined oviposition preferences to their daughters—complicates assessments of this vulnerable species. The maternal inheritance of mitochondrial DNA sequences makes CO1 an excellent gene to determine if genetically distinct host plant races have evolved in frosted elfins. In this paper, we extracted DNA using cast larval skins, a non-lethal, minimal-disturbance method appropriate for insects of conservation concern. Fifty eggs and caterpillars were taken from the field, reared in the lab until molting, and then returned to the plant on which they were found. Over 80 % of individuals had DNA successfully sequenced from their cast larval skins. The sequences allowed unequivocal identification. Neither the lupine-feeding nor wild indigo-feeding populations formed monophyletic clusters because many lupine-feeding and wild-indigo feeding individuals shared the same CO1 658 base pair sequence. An isolated population from the mountains of western Maryland was also not genetically distinct from a coastal population 345 km to the east. These results show the usefulness of using cast larval skins as a non-lethal source of DNA in listed species and suggest that frosted elfins are generalist feeders of lupine and wild indigo and are not comprised of two genetically distinct host plant races.

Keywords *Callophrys irus* · Wild indigo · Lupine · Host plant races · Mitochondrial CO1 DNA barcodes · Deer

✉ Jennifer A. Frye
Jennifer.frye@maryland.gov

Robert K. Robbins
RobbinsR@SI.edu

[1] Maryland Department of Natural Resources, Natural Heritage Program, 909 Wye Mills Rd., Wye Mills, MD 21601, USA

[2] Department of Entomology, Smithsonian Institution, NHB Stop 105, PO Box 37012, Washington, DC 20013-7012, USA

Introduction

The vulnerable North American frosted elfin butterfly (*Callophrys irus* [Godart], Lycaenidae) utilizes both lupine (*Lupinus perennis* L.) and wild indigo (*Baptisia tinctoria* (L.) Vent) as caterpillar host plants. Larval behavior is different on each plant. Caterpillars on lupine eat flowers and seed pods (NatureServe 2014; Pfitsch and Williams 2009; Schweitzer 1992a, b; Swengel 1996; Fig. 1a). Caterpillars on wild indigo eat new foliage (Fig. 1b), and are often found resting at the base of the plant, where they partially "girdle" the stem (Albanese et al. 2007a; Fig. 1d, f). Gatrelle (1991) reported that the adults of the host races differ morphologically and represent different taxa. Frosted elfin populations at any locality reportedly use one plant or the other (NatureServe 2014; Schweitzer 1992a, b), and papers on frosted elfin ecology, including restoration, have focused exclusively on lupine-feeding (Bried et al. 2012; Pfitsch and Williams 2009; Swengel 1996) or wild indigo-feeding populations (Albanese et al. 2007b, 2008). Finally, adults of the lupine-feeding races purportedly emerge about 10 days earlier than the wild indigo-feeding races

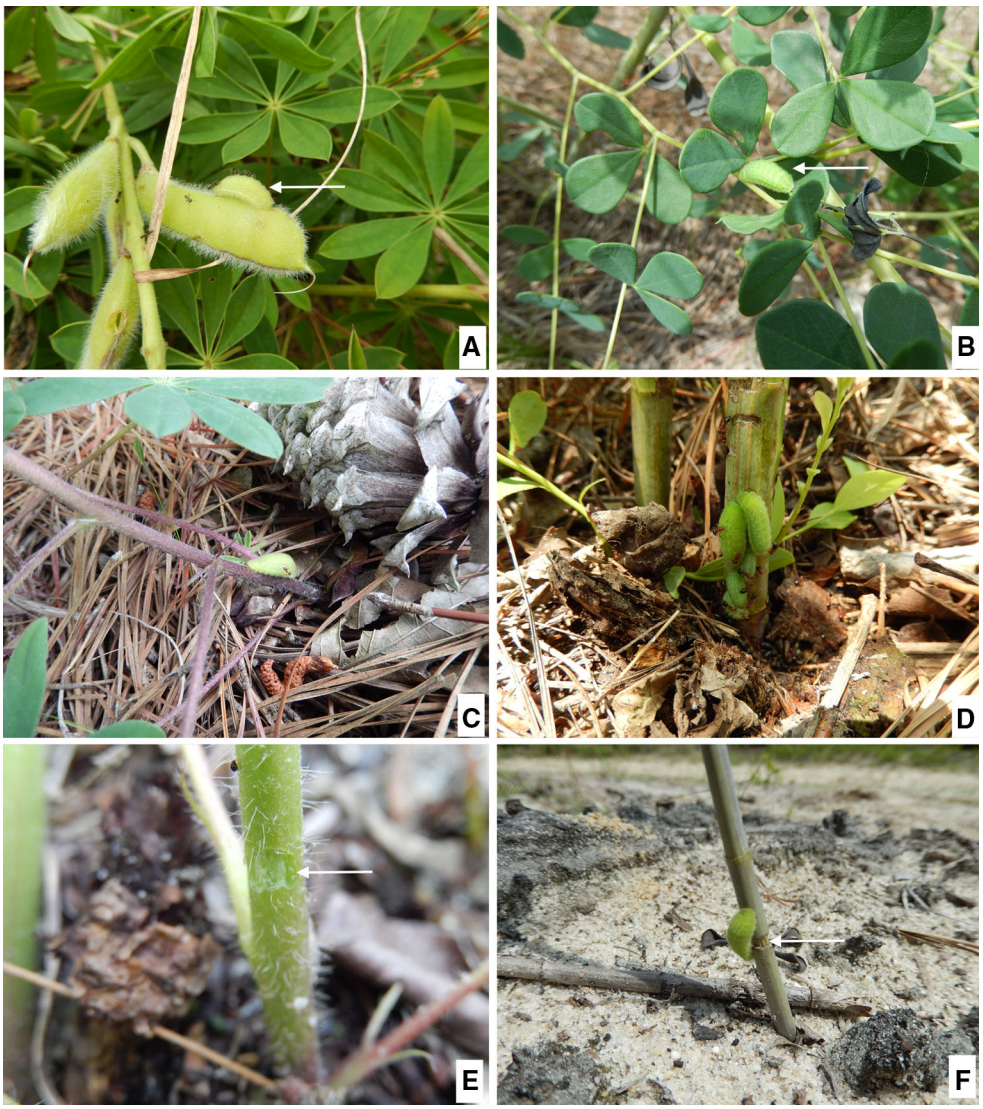


Fig. 1 Caterpillars at Study Site 1 on lupine and wild indigo. **a** Caterpillar (*arrow*) on lupine seed pod with tending ants. **b** Caterpillar (*arrow*) on wild indigo terminal shoot. **c** Caterpillar at base of lupine with tending ant. **d** Caterpillars at base of wild indigo with tending ants. **e** Girdled stem (*arrow*) of lupine. **f** Girdled stem (*arrow*) of wild indigo with larva

(Schweitzer 1992a). These apparent differences have led to the suggestion that frosted elfins are comprised of two genetically distinct host plant races (NatureServe 2014; Schweitzer 1992a), although others have noted the lack of persuasive evidence (Schweitzer et al. 2011).

The possibility of two frosted elfin host plant races is significant. The frosted elfin is a globally rare species that is critically imperiled or threatened in virtually every state where it occurs in the United States, including Maryland (Maryland NHP 2010), and is extirpated in Maine, Illinois, and Ontario, Canada (NatureServe 2014). If the butterfly is composed of two genetically distinct host plant races, conserving two genetic races will be a greater challenge than conserving one and would necessitate a reassessment of its conservation status. The more general question is whether a phytophagous species with more than one food plant is a generalist on those plants or develops genetically specialized host plant races. Such genetically host plant races were early evidence for sympatric speciation (Bush 1969, 1975), and remain a subject of intense investigation and controversy (e.g., Servedio et al. 2011; Via 2001). Indeed, if a female frosted elfin possesses a heritable preference for oviposition on one of the host plants, then host plant races of frosted elfins could represent incipient species. The recent discovery of a site in Maryland (USA) where frosted elfins feed on both lupine and wild indigo (Frye and Tangren 2013) provides an opportunity to determine if host plant races have evolved, and if so, the extent to which speciation may have proceeded.

In this paper we examine DNA sequences from the mitochondrial CO1 gene as a maternally inherited proxy for female oviposition behavior that logically precedes the differentiation of host plant races. CO1 gene sequences are widely used to differentiate closely related species (Janzen et al. 2009), and are reliably extracted from insect larval and pupal remains (Hrcek et al. 2011, 2013). If it were possible to extract gene sequences from cast larval skins, one could take a caterpillar from the field to the lab, wait for it to molt (providing a cast larval skin), and return the caterpillar to the same plant in the field where it was found. This minimum-disturbance, non-lethal sampling protocol was proposed by Watts et al. (2005) and potentially is a great advantage when working with listed insect species.

Finally, to broaden the scope of our results, we compare DNA sequence results at the primary study site with those at other sites in Maryland where frosted elfins, lupine, and/or wild indigo occur.

Materials and methods

Study sites

Four of five study sites were located on the Atlantic Coastal Plain of Maryland in Worcester, Wicomico, St. Mary's, and Caroline Counties while the fifth was in the mountains of western Maryland in Garrett County (Fig. 2; Table 1). Exact locations are not given, in accord with a request from the Maryland Department of Natural Resources (DNR) to help protect these colonies. We summarize the primary characteristics of each site in Table 1. We found no frosted elfins in other areas in Maryland with lupine and/or wild indigo, including areas where frosted elfins had occurred or were thought to occur because lycaenid larvae had been observed on lupine.

Study Site 1 was the primary locality where we conducted the project, for which reason we provide more detailed information about this site. It contains extensive clusters of lupine, primarily in areas that were clear-cut in 2004. Isolated lupine plants are also unevenly dispersed throughout much of the site. Wild indigo plants are not nearly as clustered and tend to occur in a more linear pattern along sandy roadsides, with as many as 30 or more plants in the larger stands. Lupine and wild indigo grew within 5 m of each other in four places in 2014, but were otherwise separated by greater distances.

It was recently documented that deer were eating lupine flowers and seed pods at Study Site 1 (Frye 2012). For this reason, a temporary electric fence was erected around a ~0.4 hectare area of the central lupine cluster for approximately 13 weeks during flowering and fruiting in 2013 and 2014. Occasional damage to wild indigo may have been caused by deer, but we could not be certain of this. In any case, the damage to wild indigo was significantly less than to the lupine flowers and seed pods outside the fence.

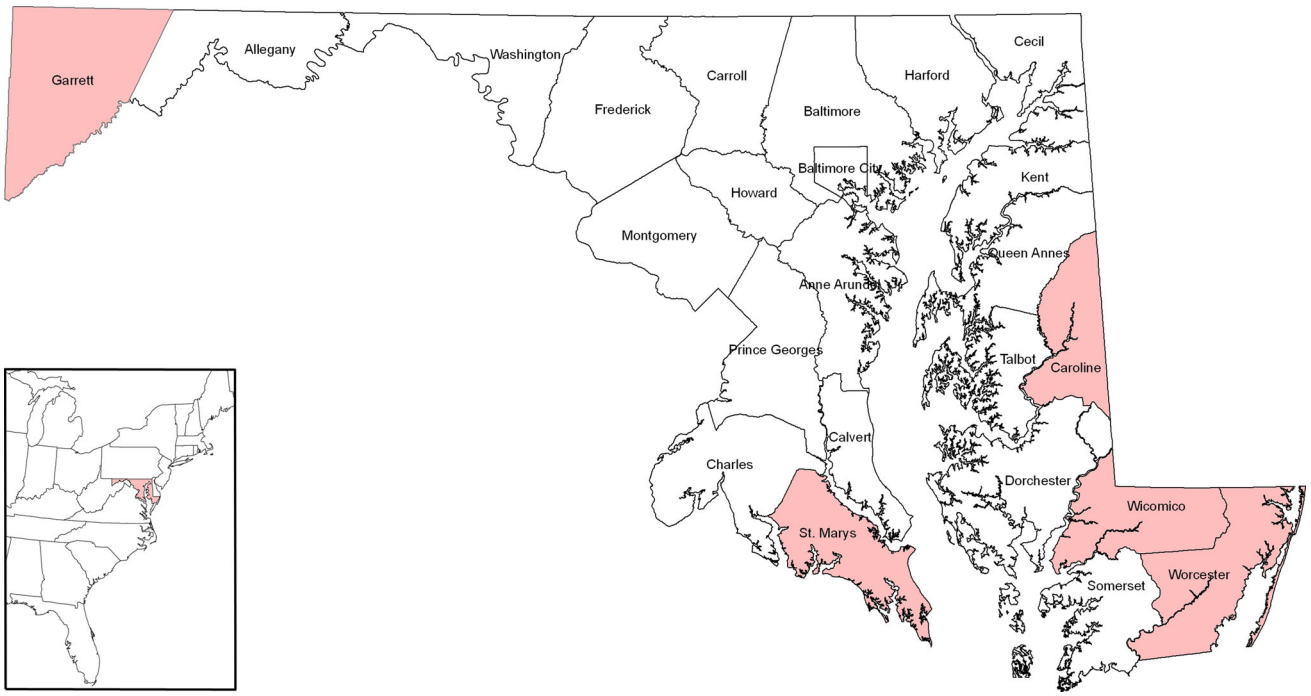


Fig. 2 Maryland county map. Field sites located in *pink-shaded* counties (*inset* the eastern United States with Maryland *pink-shaded*). *Colors* refer to online version only

Table 1 Summary information on study sites

Study site #	County	Size	Further details	Visits in 2014	Plants	Cast larval skins
1	Worcester	~18 ha	Frye and Tangren (2013)	19	Lupine and wild indigo (see text)	39
2	Worcester Wicomico	3.5 km along a dirt road	Frye and Tangren (2013)	5	Lupine and wild indigo	6
3	Caroline	~0.7 ha		5	Lupine	2
4	St. Mary's	~8.4 ha		1	Wild indigo	0
5	Garrett	~10 ha		1	Wild indigo	3

Sampling

We searched for caterpillars at the five study sites in May and June 2014. On lupine, we looked for larvae on flowers and seed pods (Fig. 1a), but also found larvae at the base of the plants (Fig. 1c). Frosted elfin caterpillars are often tended by ants of a variety of species (Fig. 1a, c), so we also looked for ant activity. On wild indigo, we searched terminal shoots and the base of the plants (Fig. 1b, d, f). As with lupine, it was often useful to look for ant activity (Fig. 1d). At Study Site 5, we looked for eggs on the wild indigo because we found no larvae.

We prepared petri dishes for caterpillar collection and transport following the protocol in Chew (1980). In brief, plastic 10 cm diameter petri dishes were half filled with commercially available plaster of paris (gypsum). After drying overnight, 9 cm diameter filter paper circles were placed on the plaster of paris. The petri dishes were squirted with water to provide a moisture reservoir.

We assigned a sample number to each caterpillar found in the field (as well as to each of the three eggs found at Study Site 5). The plant on which the caterpillar was feeding was marked with a flag with the sample number. Each larva was placed in a petri dish in the field, along with plant parts on which it was feeding. The petri dish was labeled with a unique sample number and was transported to the lab.

Caterpillars were kept in the lab with windows and no artificial light so that the photoperiod was the same as in the field. Caterpillars were checked daily. If they were feeding and frass was being produced, they were given more food as needed. If they stopped feeding, which usually occurred 1–3 days before molting, we checked for a cast head capsule, which we used as the definitive indication of molting. After finding a head capsule, we placed the cast larval skin, which is not eaten by frosted elfin larvae (at least in later instars), in a vial with the sample number. The caterpillar was then returned to the plant in the field on which it had been collected. In two cases with early instar larvae, the cast larval skin was so small (or was possibly eaten) that it was necessary to wait until it had molted a second time. In many cases, a fourth (last) instar larva pupated. We placed pupae at the base of the plant on which we had previously found the last instar. Three caterpillars from Study Site 5 developed a fungus and died. These were placed in a labeled vial with 95 % ethanol in preparation for DNA sequencing.

Comparison of DNA sequences with those of co-occurring Lycaenidae was important because identification characters for North American lycaenid larvae are largely unknown (but see the excellent work on last instars of Californian lycaenids in Ballmer and Pratt 1988). For this reason, we sampled additional Lycaenidae at Study Sites 1 and 2 including one adult each of Henry's elfin (*Callophrys henrici* [Grote and Robinson]), pine elfin (*Callophrys niphon* [Hübner]), and eastern tailed blue (*Everes comyntas* [Godart]). We removed a leg for DNA sequencing from each of these adult vouchers, which were deposited in the USNM collection (National Museum of Natural History, Smithsonian Institution, Washington, DC). The only other Lycaenidae observed at the study sites during the sampling period was the gray hairstreak (*Strymon melinus* Hübner). We added three publically available CO1 sequences for eastern North American gray hairstreaks (North Carolina, Tennessee) from the Bold Database (Ratnasingham and Hebert 2007) to the analyses.

Sequencing and analysis

Cast larval skins from 47 caterpillars, three (diseased) larvae, and legs from three adults of co-occurring Lycaenidae were prepared for CO1 sequencing according to the protocol outlined in Wilson (2012), which also details each of the steps in sequencing the CO1 gene. Samples that were successfully sequenced are listed in Table 2 with their Bold process number (Ratnasingham and Hebert 2007) and GenBank accession number (http://www.ncbi.nlm.nih.gov/genbank, accessed November 2014).

For species identification, we visualized CO1 DNA sequences phenetically using the nearest neighbor joining methods in the BOLD database (Ratnasingham and Hebert

Table 2 List of successfully sequenced samples by species, food plant, and study site

Species	Life stage	Larval host plant	Locality	Bold process #	GenBank Accession #
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM201	KP150264
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM202	KP150265
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM197	KP150280
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM203	KP150299
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM199	KP150270
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM196	KP150271
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM205	KP150275
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM194	KP150288
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM204	KP150298
Callophrys irus	Cast larval skin	Lupine	Study Site 1	EUM198	KP150304
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM229	KP150263
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM236	KP150268
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM222	KP150283
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM240	KP150290
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM214	KP150294
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM216	KP150302
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM224	KP150261
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM231	KP150266
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM223	KP150272
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM218	KP150274
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM228	KP150278
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM234	KP150281
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM232	KP150285
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM219	KP150287
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM230	KP150289
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM233	KP150291
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM225	KP150292
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM221	KP150295
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM217	KP150300
Callophrys irus	Cast larval skin	Wild indigo	Study Site 1	EUM220	KP150303
Callophrys irus	Egg/larva	Wild indigo	Study Site 5	EUM241	KP150277
Callophrys irus	Egg/larva	Wild indigo	Study Site 5	EUM242	KP150269
Callophrys irus	Egg/larva	Wild indigo	Study Site 5	EUM243	KP150284
Callophrys henrici	Adult leg	Not applicable	Study Site 2	EUM192	KP150273
Callophrys niphon	Adult leg	Not applicable	Study Site 1	EUM191	KP150297
Strymon melinus	Cast larval skin	Lupine	Study Site 2	EUM208	KP150267
Strymon melinus	Cast larval skin	Lupine	Study Site 2	EUM211	KP150276
Strymon melinus	Cast larval skin	Lupine	Study Site 2	EUM207	KP150282
Strymon melinus	Cast larval skin	Lupine	Study Site 2	EUM210	KP150286
Strymon melinus	Cast larval skin	Lupine	Study Site 2	EUM206	KP150296
Strymon melinus	Cast larval skin	Lupine	Study Site 2	EUM209	KP150301
Strymon melinus	Cast larval skin	Lupine	Study Site 3	EUM212	KP150262
Strymon melinus	Cast larval skin	Lupine	Study Site 3	EUM213	KP150279
Strymon melinus	Adult	Not applicable	Tennessee	LGSM840-04	GU090202
Strymon melinus	Adult	Not applicable	Tennessee	LGSM839-04	GU090203

Table 2 continued

Species	Life stage	Larval host plant	Locality	Bold process #	GenBank Accession #
Strymon melinus	Adult	Not applicable	North Carolina	LGSMD670-05	GU088495
Everes comyntas	Adult leg	Not applicable	Study Site 1	EUM193	KP150293

The species are frosted elfin (*C. irus*), Henry's elfin (*C. henrici*), pine elfin (*C. niphon*), gray hairstreak (*S. melinus*), and eastern tailed blue (*E. comyntas*)

2007), which produces a phenogram of distance relationships. To compare sequence similarity, we downloaded the CO1 sequences. To determine the phylogenetic distinctiveness of genetic host races, we analyzed them phylogenetically using maximum parsimony in TNT (Goloboff et al. 2008) and maximum likelihood in Garli (Zwickl 2006). Phylogenetic relationships and character state changes were illustrated in WinClada software (Nixon 2002) using the "unambiguous changes only" option. For the phylogenetic analyses, the eastern tailed blue in the Polyommatinae was used as the outgroup because the remaining species belong to the Theclinae.

Results

Sampling

At Study Site 1, adults of frosted elfins were common to abundant, as in past years (Frye and Tangren 2013). Females landed on flower stalks of lupine and on small terminal leaves of wild indigo and bent their abdomens as if they were ovipositing. Upon checking the lupine and wild indigo more carefully, we found eggs in many instances where females exhibited such behavior. However, following individual females of frosted elfins for more than a very short period of time was extremely difficult, even with a group of volunteers, because of the small size of the butterfly coupled with its rapid flight around dense scrub vegetation. At this study site, we also observed adult pine elfins, Henry's elfins, and eastern tailed blues flying in the vicinity of both lupine and wild indigo. We saw several adult gray hairstreaks on lupine plants.

We collected and assigned sample numbers to 12 lupine-feeding and 27 wild indigo-feeding lycaenid caterpillars at Study Site 1 (Table 1). Cast larval skins were obtained from these 39 larvae.

At Study Site 2, we observed no adult frosted elfins, but Henry's elfin was seen regularly. We found no caterpillars on wild indigo. However, we collected and assigned sample numbers to six lycaenid caterpillars found on lupine seed pods or at the base of the stems (Table 1). Cast larval skins were obtained from these larvae.

At Study Site 3, we observed no adult frosted elfins or other Lycaenidae. We collected and assigned sample numbers to two lycaenid caterpillars feeding on lupine flowers and seed pods (Table 1). Cast larval skins were obtained from these larvae.

At Study Site 4, we observed no adult frosted elfins nor did we find caterpillars on wild indigo despite searching well over two hundred plants. This negative result was unexpected because we identified an adult male museum specimen of a frosted elfin that was collected at Study Site 4 in 1997.

At Study Site 5, adult frosted elfins have been observed periodically by Maryland DNR staff since 1991. No adult frosted elfins were observed in 2014, but we collected and assigned sample numbers to three lycaenid eggs on wild indigo (Table 1). The resulting larvae developed a fungus and died, but we used the larvae to obtain DNA sequences.

Sequencing, identification, and relationships

At Study Site 1, two adults (leg) and 30 of 39 cast larval skins were successfully sequenced. From Study Sites 2, 3, and 5, eight cast larval skin specimens, three larvae (diseased), and one adult (leg) were successfully sequenced (sequence length >500 base pairs) (Table 2). There was no evident pattern among the nine from which DNA sequences were not recovered. These samples included caterpillars from lupine and wild indigo, from early and late instar larvae, and from caterpillars sampled on different dates.

The cladogram of relationships among specimens from this project plus the publically available sequences for the gray hairstreak provided clear-cut identification in that species clumped tightly together (Fig. 3). All larval samples from Study Sites 2 and 3 were gray hairstreaks eating lupine. All larval samples from Study Sites 1 and 5 were frosted elfins.

There were two CO1 genotypes for the frosted elfins in this study. The 20 samples in the bottom cluster (Fig. 3) have exactly the same 658 base pair sequence except for one sample in which five nucleotide positions did not code. These 20 samples include lupine-feeders and wild indigo-feeders. The 13 samples in the upper cluster also have exactly the same 658 base pair sequence (Fig. 3; Table 2) except for nucleotide positions that did not code in two individuals. Similarly, they include lupine-feeders and wild indigo-feeders, as well as samples from Study Sites 1 (coastal lowland) and 5 (inland montane).

The TNT shortest tree (maximum parsimony) and Garli best tree (maximum likelihood) had the same topology

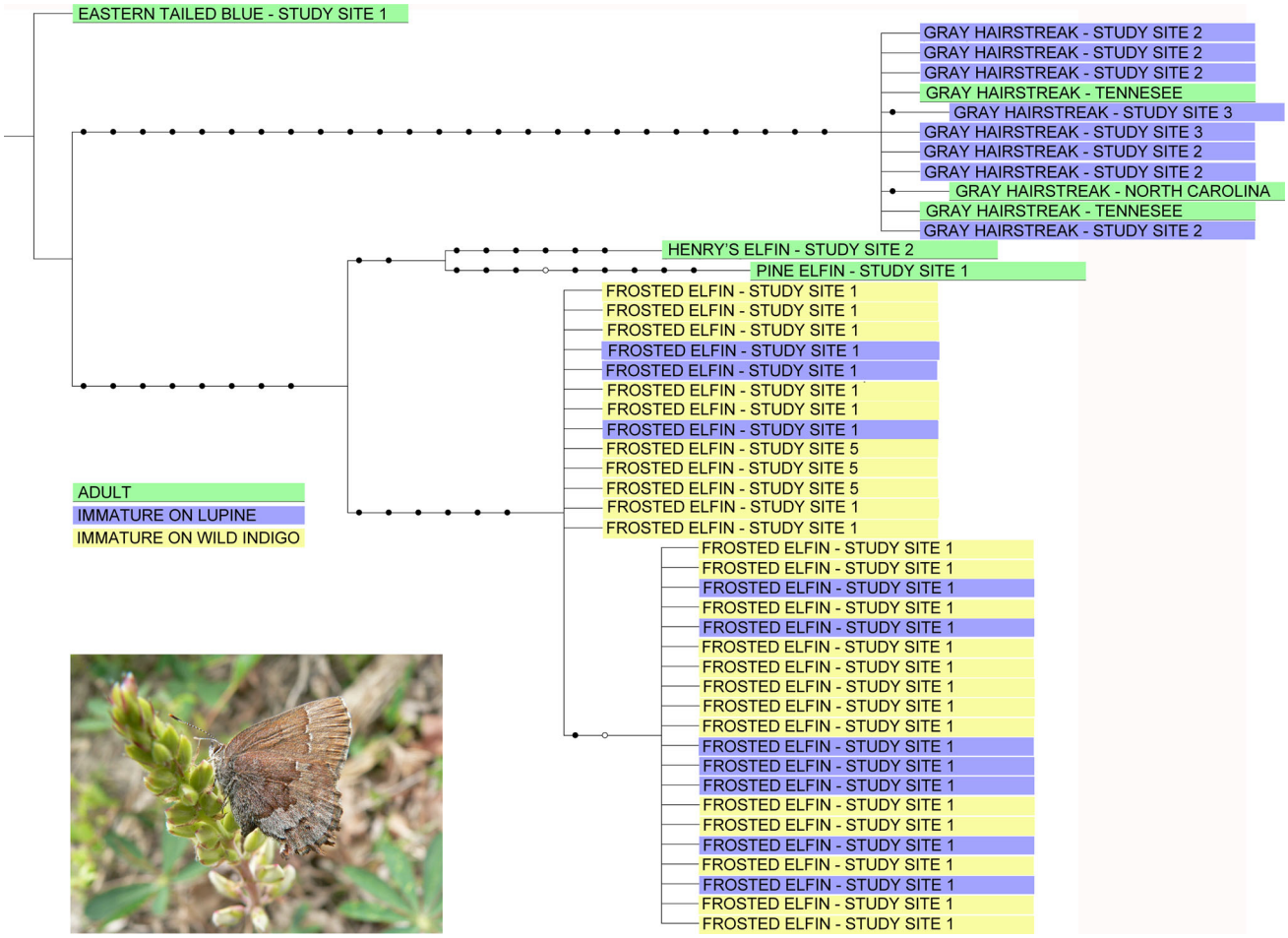


Fig. 3 Maximum parsimony tree. Neither the lupine-feeding (*blue*) nor wild indigo-feeding (*yellow*) frosted elfins formed a monophyletic cluster. Neither the individuals from western Maryland (Study Site 5, 725 m elevation) nor those from eastern Maryland (Study Site 1, 8–15 m elevation) formed a monophyletic cluster. The number of unambiguous base pair changes is shown by *solid circles* (*hollow circles* are homoplastic changes). The best maximum likelihood tree had the same topology. *Inset* is an adult frosted elfin. *Colors* refer to online version only

(Fig. 3). Neither the lupine nor the wild indigo feeders formed monophyletic clusters. Neither frosted elfins from Study Site 1 or Study Site 5 formed monophyletic clusters.

The lineage on the bottom is characterized by two nucleotide sequence synapomorphies. One is a third position "nonsense" change from TAT to TAC, both of which code for tyrosine. The other is a second position change from CCT (codes for proline) to CTT (codes for leucine).

Discussion

Cast larval skins as a source of DNA

Using cast larval skins to obtain mitochondrial DNA sequences from frosted elfins was largely successful with 80.9 % of specimens successfully sequenced. Microsatellite and mitochondrial DNA had previously been extracted from insect exuviae (Hrcek et al. 2011, 2013; Watts et al. 2005) with similar rates of success in obtaining sequences. The difference in the methodology is that frosted elfin are of conservation concern, and we returned the larvae or pupae to the plant on which they had been found.

There are two counteracting factors that might be investigated in the future. First, caterpillars are protected from parasitoids while they are in the lab, so the procedure used might decrease mortality. Alternately, bringing larvae or pupae to the lab and returning them to the field might increase their mortality due to handling or other factors. In this project, three immatures that were sampled as eggs at Study Site 5 developed a fungus in the lab (presumably diseased), but this occurs with some regularity in our experience with lycaenid larvae (e.g., Robbins 1991; Robbins and Aiello 1982).

On the basis of current information, this non-lethal methodology, which was first suggested by Watts et al. (2005), could be applied easily to other insect species of

conservation concern if DNA sequences are needed to answer questions.

Host plant races

Evidence from CO1 DNA sequences does not support the hypothesis that frosted elfins consist of two host plant races in Maryland. Neither the lupine feeding nor wild indigo feeding caterpillars formed a monophyletic cluster, as would be expected if there were maternally inherited host plant races. More importantly, the bottom lineage of 20 frosted elfin samples (Fig. 3) is characterized by two synapomorphies. If these 20 samples represent descendants through maternal inheritance from an ancestral female, then oviposition specificity is not genetically determined because the lineage includes both lupine and wild indigo feeders. For the host plant race hypothesis to be viable, the samples in the bottom lineage had to be inherited from at least two females in which there were mutations in exactly the same two of 658 nucleotide positions combined with no changes in any other nucleotide. Further, the first mutation would have to be from TAT to TAC and the second from CCT to CTT. While the first does not alter the coded amino acid (a third position "nonsense" codon), the second changes a coded proline amino acid to a leucine. This scenario is possible, but unlikely.

Behavioral flexibility

We infer from our results that frosted elfin adult females have "flexible" oviposition behavior, but this behavior could take different forms. At one extreme, it is possible that once a female lays an egg on one of the potential food plants, she will lay eggs only on that plant. At the other extreme, she may lay eggs on either plant as she encounters suitable oviposition sites. As noted, it was impractical to follow individual females in the field for time periods that were sufficient to determine the details of female frosted elfin oviposition behavior. We are skeptical that these details can be feasibly determined at Study Site 1.

We also infer from our results that frosted elfin caterpillars can eat either lupine or wild indigo. Some feeding behaviors that were reported from one food plant have now been observed from both. For example, we typically found larvae at the base of both host plants. Further, both lupine and wild indigo at Study Site 1 were sometimes girdled (Fig. 1e, f). Although it was not always clear which caterpillar had girdled the plant, girdling behavior among eumaeine lycaenids is reported only in frosted elfins. Finally, one last instar frosted elfin ate mostly lupine leaves in the lab even when it was offered pods—it successfully pupated. Previously, leaf feeding was reported only from wild indigo.

Despite these observations, "flexible" feeding behavior in frosted elfin caterpillars is still an open question. While it is feasible to find caterpillars in the field and switch half of them to the other food plant to see if feeding behavior changes, we suspect that such an experiment would be inconclusive and unwise. Insects tended by ants may "cloak" themselves with the odor of the plant on which they are feeding (Silveira et al. 2010) so that the ants treat these insects as part of the plant and do not attack them. If this were the case for frosted elfins, then switching a larva from lupine to wild indigo, or vice versa, could expose the caterpillar to an ant attack. Alternately, eggs from one female could be split between the two food plants and the feeding behavior of the hatched larvae could be observed. We did not do this experiment in 2014 for two reasons. First, if there were genetically distinct host plant races, behavioral flexibility of caterpillars on a plant that they would not naturally encounter would be a moot point. Second, we were leery of capturing adult females of a listed species for oviposition in the lab because it might alter their behavior upon release. In the future, it may be possible to take eggs from the field and switch some of them to the other food plant to determine behavioral flexibility in larvae.

Other conservation implications

Most eastern North American butterfly species that eat only lupine, including the endangered Karner blue (*Lycaeides melissa samuelis* Nabokov), have sharply declined (Schweitzer 1992a; Wagner et al. 2003). Browsing by deer is one potential causative factor discussed in Schweitzer et al. (2011). Temporary electric fencing was used successfully at Study Site 1 during lupine flowering and fruiting in 2013 and 2014 to prevent damage from deer browse. These observations, along with the results in Frye (2012), suggest that this precaution is one that might be more widely adopted. Those working with lupine-feeding insects in areas that are heavily impacted by deer may want to consider a similar course of action.

Frosted elfins appear to be of greater conservation concern in Maryland than may have been realized. The number of localities in Maryland with "large" stands or a "large" number of small patches of lupine or wild indigo is limited, which sets an upper limit to the number of frosted elfin populations. However, we did not find frosted elfin immatures or adults at other localities where they had been reported or where they were suspected to occur. Study Site 2 has both food plants and is only 7 km from Study Site 1, but we found no adults or larvae of frosted elfins there. We do not know if Study Site 3 has sufficient lupine to support a population of frosted elfins, but in any case we did not find the butterfly there. Finally, we know from a museum specimen that frosted elfins occurred at Study Site 4 in 1997. The stands of wild indigo at this site are much larger

than at Study Site 1, but we found no evidence of adults or caterpillars in 2014 despite extensive searching. Negative evidence needs to be treated with caution—and in 2015 frosted elfins were reported at two additional localities near Study Site 1—but these results suggest that frosted elfins in Maryland may be more vulnerable than had been thought.

Acknowledgments We thank the following individuals for their assistance in all aspects of field work: Paula Becker, Danny Thomas, Chris Frye, John Moulis, Kyle Rambo, Wes Knapp, Joe Fehrer Sara Tangren, and numerous volunteers from the Maryland DNR and the Nature Conservancy, most notably Jeff Bacon, Gordon and Mary Burton, David Hindle, Gary Marine, Tom Ogden, Margaret Schultz, Robert Turk and Tom Ogden. We thank Brian Harris and Margaret Rosati at Smithsonian for technical support, and Scott Miller (who urged us to use cast larval skins as a source for mitochondrial DNA), Paul Goldstein, and Karie Darrow for helpful advice. Dana Limpert at Maryland DNR provided mapmaking expertise, and Jeremy deWaard at Guelph greatly facilitated the sequencing of our samples. For reviewing and commenting on the manuscript, we are grateful to Robert Busby, Paul Goldstein, Scott Miller, Richard Smith, and David Wagner. Two anonymous reviewers and the Editor made many helpful suggestions, for which we are grateful. This project was funded in part through a State Wildlife Grant from the US Fish and Wildlife Service, and through the Department of Entomology, NMNH, Smithsonian Institution.

References

Albanese G, Nelson MW, Vickery PD, Sievert PR (2007a) Larval feeding behavior and ant association in frosted elfin, *Callophrys irus* (Lycaenidae). J Lepid Soc 61:61–66

Albanese G, Vickery PD, Sievert PR (2007b) Habitat characteristics of adult frosted elfins (*Callophrys irus*) in sandplain communities of southeastern Massachusetts, USA. Biol Conserv 136:53–64

Albanese G, Vickery PD, Sievert PR (2008) Microhabitat use by larvae and females of a rare barrens butterfly, frosted elfin (*Callophrys irus*). J Insect Conserv 12:603–615

Ballmer GR, Pratt GF (1988) A survey of the last instar larvae of the Lycaenidae of California. J Res Lepid 27:1–81

Bried JT, Murtaugh JE, Dillon AM (2012) Local distribution factors and sampling effort guidelines for the rare frosted elfin butterfly. Northeast Nat 19:673–684

Bush GL (1969) Sympatric host race formation and speciation in frugivorous flies of the genus *Rhagoletis* (Diptera, Tephritidae). Evol 23:237–251

Bush GL (1975) Modes of animal speciation. Annu Rev Ecol Syst 6:339–364

Chew FS (1980) Foodplant preferences of *Pieris* caterpillars (Lepidoptera). Oecologia (Berl.) 46:347–353

Frye JA (2012) The effect of deer browse on sundial lupine: implications for frosted elfins. Northeast Nat 19:421–430

Frye JA, Tangren S (2013) Dual host plant use by *Callophrys irus* (Godart) (Lycaenidae) larvae at a single site on the Maryland coastal plain. News Lepid Soc 55:156–157

Gatrelle RR (1991) The taxonomic implications of the discovery of *Incisalia irus* in Florida. News Lepid Soc 1991:57–58

Goloboff PA, Farris JS, Nixon KC (2008) TNT, a free program for phylogenetic analysis. Cladistics 24:774–786

Hrcek J, Miller SE, Quicke DL, Smith MA (2011) Molecular detection of trophic links in a complex insect host-parasitoid food web. Mol Ecol Resour 11:786–794

Hrcek J, Miller SE, Whitfield JB, Shima H, Novotny V (2013) Parasitism rate, parasitoid community composition and host specificity on exposed and semi-concealed caterpillars from a tropical rainforest. Oecologia 173:521–532

Janzen DH, Hallwachs W, Blandin P, Burns JM, Cadiou J-M, Chacon I, Dapkey T, Deans AR, Epstein ME, Espinoza B, Franclemont JG, Haber WA, Hajibabaei M, Hall JPW, Hebert PDN, Gauld ID, Harvey DJ, Hasumann A, Kitching IJ, Lafontaine D, Landry J-F, Lemaire C, Miller JY, Miller JS, Miller L, Miller SE, Montero J, Munroe E, Green SR, Ratnasingham S, Rawlins JE, Robbins RK, Rodriguez JJ, Rougerie R, Sharkey MJ, Smith MA, Solis MA, Sullivan JB, Thiaucourt P, Wahl DB, Weller SJ, Whitfield JB, Willmott KR, Wood DM, Woodley NE, Wilson JJ (2009) Integration of DNA barcoding into an ongoing inventory of complex tropical biodiversity. Mol Ecol Resour 9(Supplement 1):1–26

Maryland Natural Heritage Program (2010) Rare, threatened and endangered animals of Maryland. April 2010 edition, MD Department of Natural Resources, Wildlife and Heritage Service, Annapolis, MD. 24 p 10

NatureServe Explorer (2014) An online encyclopedia of life [web application] Version 4.6. Arlington, Virginia, USA: NatureServe. http://www.natureserve.org/explorer. Accessed Nov 2014

Nixon KC (2002) WinClada ver. 1.0000. Ithaca, NY. Published by the author

Pfitsch WA, Williams EH (2009) Habitat restoration for lupine and specialist butterflies. Restor Ecol 17:226–233

Ratnasingham S, Hebert PDN (2007) BOLD: the barcode of life data system (www.barcodinglife.org). Mol Ecol Notes, p 10. Accessed Nov 2014

Robbins RK (1991) Cost and evolution of a facultative mutualism between ants and lycaenid larvae (Lepidoptera). Oikos 62:363–369

Robbins RK, Aiello A (1982) Foodplant and oviposition records for Panamanian Lycaenidae and Riodinidae. J Lepid Soc 36:65–75

Schweitzer DF (1992a) Comments regarding *Ervnnis persius persius* and *Incisalia irus*. Newsl Ohio Lepid 14:21–23

Schweitzer DF (1992b) *Incisalia irus* revisited: a response to Reverend Ronald Gatrelle. News Lepid Soc 1992:69–70

Schweitzer DF, Minno MC, Wagner DL (2011) Rare, declining and poorly known butterflies and moths (Lepidoptera) of forests and woodlands in the Eastern United States. Forest Health Technology Enterprise Team. p 517

Servedio MR, Doorn GSV, Kopp M, Frame AM, Nosil P (2011) Magic traits in speciation: 'Magic' but not rare? Trends Ecol Evol 26:389–397

Silveira HCP, Oliveira PS, Trigo JR (2010) Attracting predators without falling prey: chemical camouflage protects honeydew-producing treehoppers from ant predation. Am Nat 175:261–268

Swengel AB (1996) Observations of *Incisalia irus* (Lepidoptera: Lycaenidae) in central Wisconsin 1988–1995. Great Lakes Entomol 29:47–62

Via S (2001) Sympatric speciation in animals: the ugly duckling grows up. Trends Ecol Evol 16:381–390

Wagner DL, Nelson MW, Schweitzer DF (2003) Shrubland Lepidoptera of southern New England and southeastern New York: ecology, conservation, and management. For Ecol Manag 185:95–112

Watts PC, Daguet C, Thompson DJ, Kemp SJ (2005) Exuviae as a reliable source of DNA for population-genetic analysis of odonates. Odonatologica 34:183–187

Wilson JJ (2012) DNA barcodes for insects (Chap 3). In: Kress WJ, Erickson DL (eds) DNA barcodes: methods and protocols. Methods and Molecular Biology, vol 858, pp 17–46. Humana Press, New York

Zwickl DJ (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Dissertation, University of Texas at Austin