Systematics of the *Argyrotaenia franciscana* (Lepidoptera: Tortricidae) Species Group: Evidence from Mitochondrial DNA

BERNARD LANDRY, JERRY A. POWELL, AND FELIX A. H. SPERLING

ESPM-Insect Biology, 201 Wellman Hall, University of California, Berkeley, CA 94720

ABSTRACT Moths of the *Argyrotaenia franciscana* species group represent a challenging case of evolutionary lability and taxonomic complexity in California. We studied their evolutionary relationships using mitochondrial DNA (mtDNA) sequences from 40 specimens in 18 populations of the *A. franciscana* group, as well as 2 outgroup species. Most specimens were sequenced over a 799-bp segment of the cytochrome oxidase subunit I (COI) gene. Single specimens each of *A. franciscana insulana* Powell and *A. citrana* (Fernald) were sequenced over a 2.3-kb region including COI, tRNA leucine (UUR), and cytochrome oxidase subunit II (COII). mtDNA variation within and among *Argyrotaenia citrana, A. franciscana* (Walsingham), and *A. franciscana insulana* is most simply interpreted as DNA polymorphism within a single species for which the oldest name is *A. franciscana*. Maximal divergence among haplotypes was 3.8%, which is on the high end of the range for intraspecific mtDNA variation in Lepidoptera. *Argyrotaenia niscana* (Kearfott) is most closely related to a new species, and this pair forms the closest outgroup to the *A. franciscana-citrana* complex. The status of *A. isolatissima* Powell remains uncertain.

KEY WORDS *Argyrotaenia citrana*, apple skinworm, hybridization, California Channel Islands

STUDIES OF MITOCOCHONDRIAL DNA variation have proven helpful in understanding relationships among closely related species of Lepidoptera (e.g., Bogdanowicz et al. 1993, Sperling 1993, Brown et al. 1994, Brower 1994, Sperling and Hickey 1994, Miller et al. 1997). Such studies are more meaningful when other data are available for comparison, whether morphological, ecological, allozymic, or from hybridization. We describe a study where information on morphology, geographic distribution over time, and hybridization trials was abundant before analysis of mitochondrial DNA (mtDNA) variation.

The *Argyrotaenia franciscana* species group comprises a series of morphologically variable populations that occur along the Pacific Coast of North America from southern British Columbia to northern Baja California. Taxonomic interpretations of species or races seem to refer to 3 distinct species having differing phenotypes and habitats. Beginning in the 1920s, however, a larval pest of commercial apples in coastal Santa Cruz County and inland in Sonoma County was identified as *A. franciscana*. Meanwhile, *ci-

---

1 Current address: Agriculture Canada, ECORC, CEF, Neatby Building, Ottawa, ON, Canada K1A 0C6. E-mail: blandry@sympatico.ca

0013-8746/99/0040-0046$02.00/0 © 1999 Entomological Society of America
citrana, which had been introduced from southern California or had spread from more inland populations (Powell 1964, 1965).

Based primarily on phenotypic differences and assumptions of isolation, populations from the California Channel Islands were described _isolatissima_ from tiny and remote Santa Barbara Island, and _insulana_ from Anacapa Island (Powell 1964). The latter name was regarded as a subspecies of _franciscana_ and applied to populations of all the northern and westernmost islands (Powell 1985, 1994). _A. isolatissima_ is restricted to Santa Barbara Island, although there is a phenotype-similar population on the mainland at Point Conception, north of the Channel Islands.

Argyrotaenia niscana is broadly sympatric with members of the _franciscana-citrana_ complex and may be in close contact in coastal southern California, although we have not sampled populations in a zone of contact, and there are no known hybrids. Larvae of the _franciscana-citrana_ complex are polyphagous, whereas _niscana_ and a presumably closely related species, _lignitaenia_, are restricted to species of Californian endemic shrubs in the genus _Adenostoma_ (Rosaceae).

Finally, we discovered coastal populations of a smaller _Argyrotaenia_, here referred to as _Argyrotaenia_ n. sp., that lacks the pronounced sexual dimorphism typical of the _franciscana-citrana_ complex and the dark rust-red scaling that characterizes _niscana_. These are closely sympatric with populations in San Luis Obispo County interpreted to be _franciscana_ s. str. (Powell 1981) and hybrid _franciscana-citrana_ (Powell 1985, 1981, and current data), and we have seen no evidence of field hybridization. The larvae of this new entity appear to be specialists on woody Asteraceae (based on 6 field collections of larvae from _Ericameria_ and _Lessingia_, J.A.P., unpublished data). Formal description of this new species is planned at a later date, pending further morphological examinations and hybrid studies.

To aid in clarifying relationships, J.A.P. conducted cross-population hybridization tests, using reared, virgin females from widely distributed coastal and island localities. These represent populations assigned to _A. franciscana_ s. str., _A. f. insulana_, _A. citrana_, _A. niscana_, and _Argyrotaenia_ n. sp. The methods and data will be presented elsewhere, but the results can be summarized as follows: During 1979–1997, ~150 trials were attempted, including 60 that were intrapopulational, sometimes with siblings. Intrapopulation matings from _franciscana_ (Monterey County), _f. insulana_, and _citrana_ (San Francisco Bay area and San Luis Obispo counties) produced ~81% success (defined as production of viable eggs that developed fully to eclosion of 1st instars). By contrast, the rate of success was appreciably lower, ~62% (n = 79), among interpopulation trials involving the same array of populations. In some instances all or nearly all eggs developed normally, but more often only a portion (e.g., ~20–50%) developed, or partial development occurred in most of the eggs, or by earlier deposited eggs, only a portion of which developed fully.

Preliminary results of the hybridizations suggest _A. niscana_ and _A. n. sp._ are incompatible with members of the _franciscana-citrana_ complex, as had been expected from observations of field sympatry. Two trials with male _niscana_ and female _citrana_ and _insulana_ failed to produce mating; 9 trials with _A. n. sp._ and _citrana, insulana_, and their hybrid resulted in 1 mating between a male _A. n. sp._ and female _insulana_, but the eggs developed only partially (J.A.P., unpublished data).

The current study was undertaken to provide an independent line of evidence for understanding relationships within this morphologically similar group of moths. In particular, we focus on the genetic distinctness of _A. franciscana_ and _A. citrana_.

Materials and Methods

Specimens. We selected 51 specimens for study, 49 from within the _Argyrotaenia franciscana_ species group and 2 representing outgroups, _A. coloradana_ (Fernald) and _A. klotsi_ Obraztsov, both from Arizona (Table 1). There has been no phylogenetic study nor even a traditional classification of _Argyrotaenia_ species that defines species groups, so selection of outgroup species was based on similarity of male genitalia and biogeographic proximity. Samples represented _franciscana_ s. str., _franciscana insulana_, _citrana_, several populations known or believed to possess _franciscana-citrana_ hybrid characteristics, _isolatissima_, _niscana_, and _A. n. sp._ (Table 1). No recent material of _lignitaenia_ was obtained.

Specimens studied came from a selection of sites representing a large portion of the range of the species of the _A. franciscana_ group (Table 1). Where possible, we sampled 4 specimens from each site to determine the extent of sequence divergence within populations. The Washington laboratory colony originated from the Willamette Valley, Oregon (Knight 1996).

Ten of the specimens sequenced were reared from field collected larvae or on laboratory cultures fed synthetic diet. Most of the remainder were collected as adults in the field by B.L. and J.A.P. in 1995–1996, held in 15-dram plastic snap-top vials with a bit of damp cotton, and transported in a camp cooler. Live specimens were then frozen at ~70°C. In addition, 17 of the samples were pinned specimens, the oldest having been collected in 1978 (Table 1).

Specimens were identified initially by phenotype, specifically the forewing pattern. The abdomen and wings of each specimen were preserved in a gelatin capsule for confirmation of identification. Vouchers are deposited in the Essig Museum of Entomology, University of California, Berkeley.

Molecular Methods. DNA was purified using a phenol/chloroform-based extraction. Heterologous primers were used with genomic DNA template for amplification of mitochondrial segments with the polymerase chain reaction (PCR) (Saiki et al. 1988).

We mostly used general mtDNA insect primers (Liu and Beckenbach 1992, Simon et al. 1994) or primers designed previously for use with the spruce budworm
Table 1. Number of Argyrotaenia specimens sequenced, codes of haplotypes, and collection data

<table>
<thead>
<tr>
<th>No.</th>
<th>Taxon</th>
<th>Code(s)</th>
<th>Collecting locality* and year, counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A. n. sp.</td>
<td>Aci97</td>
<td>Santa Barbara I., Santa Barbara, 1986</td>
</tr>
<tr>
<td>2</td>
<td>A. isolatisimia</td>
<td>Aiso52</td>
<td>Santa Barbara I., Santa Barbara, 1986</td>
</tr>
<tr>
<td>3</td>
<td>A. niscana</td>
<td>Aisic37, 47</td>
<td>Boulder Oaks Campground, San Diego, 1991</td>
</tr>
<tr>
<td>4</td>
<td>A. niscana</td>
<td>Aisic981</td>
<td>UC Hastings Reserve, Monterey, 1998</td>
</tr>
<tr>
<td>1</td>
<td>A. coloradana</td>
<td>Acolo26</td>
<td>Arizona, Little Spring, Coconino, 1995</td>
</tr>
<tr>
<td>1</td>
<td>A. kloti</td>
<td>Aklot27</td>
<td>Arizona, Little Spring, Coconino, 1995</td>
</tr>
<tr>
<td>1</td>
<td>A. isolatisimia</td>
<td>Aiso52</td>
<td>Santa Barbara I., Santa Barbara, 1986</td>
</tr>
<tr>
<td>2</td>
<td>A. niscana</td>
<td>Aisic37, 47</td>
<td>Boulder Oaks Campground, San Diego, 1991</td>
</tr>
<tr>
<td>4</td>
<td>A. niscana</td>
<td>Aisic981</td>
<td>UC Hastings Reserve, Monterey, 1998</td>
</tr>
<tr>
<td>1</td>
<td>A. coloradana</td>
<td>Acolo26</td>
<td>Arizona, Little Spring, Coconino, 1995</td>
</tr>
<tr>
<td>1</td>
<td>A. kloti</td>
<td>Aklot27</td>
<td>Arizona, Little Spring, Coconino, 1995</td>
</tr>
</tbody>
</table>

* Except otherwise indicated, localities are in California.

(Sperling and Hickey 1994), but we also designed 5 more specific primers. Double-stranded polymerase chain reaction (PCR) product was cleaned with Milipore Ultrafree-MC filters and was sequenced directly, using Applied Biosystems automated sequencing with fluorescent dye terminators.

The mtDNA of 2 specimens, from Berkeley (Aci1) and San Miguel Island (A52), was sequenced over 2,295 bp beginning in the tRNA tyrosine gene and ending in the tRNA lysine gene. This 2.3-kb region corresponds to the region between bases 1,466 and 3,771 in Drosophila yakuba (Clary and Wolstenholme 1985) and includes the genes for COI, tRNA leucine, and COII. It was obtained by PCR amplification using the end primers TY-J-1460 (K698) 5' TAC AAT TTA TCG CCT AAA CTT CAG CC 3' and TK-N-3782 (Eva) 5' GAG ACC ATT ACT TGC TTT CAG TCA TCT 3', in combination with various internal primers. This fragment was chosen because of its proven utility (e.g., Sperling and Hickey 1994, Sperling et al. 1996) in other lepidopteran families at the taxonomic level investigated here, and because we are building a database of comparable sequences for future phylogenetic studies at higher taxonomic levels.

We chose a 799-bp segment in the COI gene to compare specimens from 15 more populations of the A. franciscana group and 1 specimen of each of the 2 outgroup species. This fragment corresponds to the 2nd half of COI between bp number 2201 and 2999. The region was amplified using the primers CI-J-2183 (Jerry) 5' CAA CAT TTA TTT TGA TTT TTI TTT GG 3' and TL2-N-3013 (Pat2) 5' TCC ATT ACA TAT TAT CTT CCA TAT TAG 3'.

Phylogenetic Analysis. Phylogenetic analysis was performed with PAUP 3.1 (Swofford 1993) using all default parameters. Variable nucleotide positions were treated as unordered characters with 1 state for each nucleotide. Sequences from A. coloradana and A. kloti were used to root the tree. The bootstrap option in PAUP was used to determine the extent of support of internal nodes; 500 iterations were performed.

Results

Sequence Variation. The 2.3-kb mtDNA sequences for Aci1 and A52 are shown in Fig. 1. There were 58 substitutions between these 2 sequences, or 2.5% divergence. Between these 2 sequences, COI had 3.0% divergence (46 substitutions), tRNA leucine 3.0% (2 substitutions), and COII 1.5% (10 substitutions). No insertions or deletions were observed. Among the protein coding genes there were 4 amino-acid replacements: leucine versus phenylalanine (bp 2500), asparagine versus aspartic acid (bp 2956), valine versus isoleucine (bp 3136), and methionine versus valine (bp 3518 and 3520). Of the 58 nucleotide substitutions, 4 were transversions, and 52 were in the 3rd position, and 6 were in the 1st position. The complete 2.3-kb fragment is composed of 39.1% T, 33.9% A, 13.9% C, and 6.0% G.

We were able to obtain 799 bp of sequence for 48 of 51 specimens selected for study. One DNA template was apparently contaminated and for 1 specimen of A. isolatisimia the DNA template did not amplify, and for the 2nd (Aiso52) we could not obtain a clean sequence between bases 2350 and 2530.

Among the 49 sequences obtained there were 28 unique haplotypes, with nucleotide variation at 131 sites (Fig. 2). The distribution of haplotypes varied among populations. For example, we found only 1 haplotype in the 4 specimens from Berkeley and in the 4 specimens from the Washington laboratory colony, possibly reflecting a restricted gene pool in these populations. A more diverse sample is represented by the unique haplotype found in each of the 3 specimens of A. franciscana from Dune Lakes, San Luis Obispo County, and in the 3 haplotypes found among the 4
specimens of the new species collected at Montana de Oro State Park, San Luis Obispo County.

For mtDNA within the *franciscana* clade (Aci1 A¢1 AÞ1 Aiso), divergence was up to 3.8% (Aci15 versus AÞ45). Between the new species and *A. niscana*, divergence was 2.6–3.3%. Between the *franciscana* clade and the new species or *A. niscana*, divergence was 5.0–6.5%. The mtDNA of *A. klotsi* was 6.5–7.5% diverged from all of the above haplotypes. *A. coloradana* was 8.3–9.3% diverged from all other haplotypes, except 7.3% from *A. klotsi*. Patterns of nucleotide substitutions in the 799-bp fragment were similar to those described for the 2 sequences of 2.3 kb.

**Phylogenetic Analysis.** In the 48 sequences of 799 bp and 1 partial sequence, 28 haplotypes were unique. None of the unique haplotypes was found at 2 localities, and 3 were found at 2 localities each (Montana de Oro and Dune Lakes, Brooks Island and Washington laboratory colony, and San Miguel Island and Santa Rosa Island). A heuristic parsimony search of the 28 haplotypes in PAUP resulted in 2 trees of 213 steps each, the topological variability being restricted to haplotypes of the new species. The bootstrap consensus tree is shown in Fig. 3 and corresponds to the consensus of the 2 most parsimonious trees.

The mtDNAs of specimens identified either as *franciscana* or *citrana* do not show any particular pattern of relationships. The mtDNAs of the California Channel Island specimens tend to cluster together, but the mtDNA of specimens with obvious *citrana* phenotype from Brooks Island, Los Angeles, and the Washington laboratory colony also cluster with them. Bootstrap support for the 2 larger *franciscana* subclades is weak (53 and 60%). Thus, the distribution of *citrana* and *franciscana* phenotypes is incongruent with that of mtDNA sequences.

However, the mtDNAs of specimens identified as *niscana* and *Argyrotaenia* n. sp. all clearly cluster together. Bootstrap values indicate maximal support (99–100%) for the monophyly of the 4 main basal clades: *A. niscana*, *A. n. sp.*, *A. n. sp. + *A. niscana*, and *A. citrana + franciscana + f. insulana + isolatissima*. This supports recognition of 3 separate species (*A. niscana*, *A. n. sp.*, and *A. franciscana*) and a sister group relationship between *A. niscana* and *A. n. sp.*
Discussion

The most commonly employed criterion for delimiting species is that they are reproductively isolated populations (Mayr 1969). In the case of *A. franciscana* and *A. citrana*, laboratory hybridization has failed to show an appreciable amount of postzygotic isolation (J.A.P., unpublished data). However, laboratory hybridization may be relatively uninformative about prezygotic isolating mechanisms that operate only under natural conditions. An alternate means to deter-

---

**Fig. 2.** Nucleotide variation in 28 unique mtDNA haplotypes of *Argyrotaenia*. Dashes in Aiso52 indicate where no sequence was obtained. Numbers above columns refer to nucleotide positions.

**Fig. 3.** Phylogram representing relationships of all haplotypes. Numbers above internodes are values of bootstrap support. Locality origins are indicated on a map of California. Specimens with identical haplotypes are shown separately, with zero horizontal branch length between them, when they are found at 2 localities (e.g., Aci5 and Aci6 versus Af46).
mine whether populations are reproducively isolated is to use molecular assays to survey for gene clusters (Mallet 1995). Our examination of the mtDNA sequence variation is a genetic survey of these species. Although our study focuses on a single locus with a relatively unusual maternal inheritance pattern, it has the advantage of being potentially quite sensitive to genetic bottlenecks (Moore 1995) and is arguably the best single indicator of species limits in Lepidoptera (Sperling 1994). Furthermore, we found no evidence of heteroplasmic, or mtDNA sequence variation, within individuals, and the relatively low genetic divergences between species are easily analyzed using phylogenetic inference. The resulting mtDNA trees lend themselves to interpretation under a phylogenetic species concept that views species as smallest detectable monophyletic population units (Nixon and Wheeler 1990).

Application of the phylogenetic species concept to Argyrotaenia mtDNA suggests that populations of A. citrana, franciscana, f. insulana, and probably also isolatissima, form 1 species. This species would be called A. franciscana, because it is the oldest available name. Sequence divergence within the main A. franciscana mtDNA lineage is relatively high. The maximal sequence divergence within this lineage (3.8%) is 0.5% higher than the maximal percent sequence divergence between A. niscana and the new species. It is also higher than results obtained in other similar studies on Lepidoptera. In a study of the same 800-bp mtDNA region in pheromone types in the dingy cutworm (Noctuidae), maximal sequence divergence within species was 2.3%, and 3.7% when specimens believed to belong to a sibling species were considered (Sperling et al. 1996). Among subspecies of the hemlock looper (Geometridae), 2.2% maximal divergence occurred in a larger segment homologous to the 2.3 kb region sequenced in single specimens of A. citrina and A. franciscana, which had a divergence of 2.5% (Sperling et al. 1999). Within the C. fumiferana species group (5 species, Tortricidae), 2.9% divergence occurred in a 1.6-kb fragment of COI and COII (Sperling and Hickey 1994). Finally, among 3 species of ermine moths (Yponomeutidae) <1% divergence occurred across the 2.3 kb COI-II region (Sperling et al. 1995). Thus, simple percent sequence divergence between closely related sister species of Lepidoptera is highly variable and is not necessarily a good predictor of whether 2 unknown populations constitute reproducively isolated species. However, it is clear that mtDNA divergences within A. franciscana+citrana are deep and presumably old.

The lack of support for monophyletic subclades for A. franciscana or A. citrana is consistent with observations of phenotypic change through time in the San Francisco Bay area and Santa Cruz Island, which suggests hybridization in disturbed habitats (Powell, 1964, 1965, and unpublished data), and is supported by laboratory hybridization trials (J.A.P., unpublished data).

Based on our data, A. franciscana insulana and A. isolatissima probably should also be synonymized with A. franciscana, but more data from the Channel Islands populations, especially from Anacapa and Santa Barbara Islands, are required to establish this conclusion more firmly. More mtDNA data on other populations, as well as studies of other genetic markers, may also help to give a better assessment of relationships within A. franciscana. Our mtDNA analysis also supports the suspected presence of a new species in San Luis Obispo County and provides clear support for a sister species relationship with A. niscana.

Acknowledgments

We thank G. Roux, A. Cognato, M. Caterino, and D. Kain who helped with molecular techniques and sequencing of specimens. We are grateful to K. Walker (UC Berkeley), who maintained a laboratory colony of A. citrana that originated from A. L. Knight (USDA, ARS, Wapato, WA), to J. P. Donahue (Los Angeles, CA), and to R. Robertson (Santa Rosa, CA) for providing specimens; also to personnel of the California State Parks, Sacramento, for a permit to sample at Montana de Oro State Park; and to J. Clegg (University of California Bodega Marine Laboratory) for permission to collect specimens in that preserve. This project was made possible by an NSF-PEET grant to F.A.H.S. and J.A.P., and by a California Agriculture Experiment Station grant to F.A.H.S.

References Cited


Received for publication 30 July 1998; accepted 8 October 1998.