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

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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 49 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 MITOCHONDRIAL 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 have varied, not only with increased understanding of named entities, but because some populations have changed in phenotypic and, presumably, genetic makeup during urbanization (Powell 1964, 1965).

The following species and subspecies names have been applied (all with type localities [TL] in California). *Argyrotaenia franciscana* (Walsingham, 1879) (TL: San Francisco); *A. citrana* (Fernald, 1889) (TL: Los Angeles); *A. niscana* (Kearfott 1907) (TL: Carmel); *A. kearfotti* Obraztsov, 1961 (TL: Carmel); *A. franciscana insulana* Powell, 1964 (TL: Anacapa Island); *A. isolatissima* Powell, 1964 (TL: Santa Barbara Island); *A. lignitaenia* Powell, 1965 (TL: Pinyon Flat, Riverside County); and "*Argyrotaenia* n. sp." Powell, 1981 (Oso Flaco Lake, San Luis Obispo County).

Argyrotaenia kearfotti was considered a subjective synonym because it is an individual phenotypic variant (Powell 1964), an assumption that has been confirmed by subsequent rearing from eggs (J.A.P., unpublished data). In addition, *Tortrix purata* Meyrick, 1932, was described from "California, Venice [Los Angeles Co.]. and Costa Rica," then transferred to *Argyrotaenia* by Freeman (1958). It is omitted from our discussion because Obraztsov (1961) selected a lectotype from the Costa Rican specimens, and the California examples (U.S. National Museum [USNM]) were confirmed as *A. citrana* (Powell 1964).

Before 1920, the earliest names, *franciscana, citrana,* and *niscana,* seemed 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 *franciscana.* Meanwhile, *citrana* in southern California, originally noticed feeding on citrus and other plants, had become a widespread pest of numerous field crops and ornamental plants. It was recognized in central California (e.g., Lange 1936), and uncertainty developed concerning the taxonomic and biological distinctness of the 2 (Bartges 1951). In fact, circumstantial evidence based on morphological and phenotypic characters indicated that coastal *franciscana* populations of agricultural and urban areas had been modified by hybridization with

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populations of all the northern ar
lands (Powell 1985, 1994). *A. isolat*
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typically similar population on the
Conception, north of the Channel
Argyrotaenia niscana is broadl
members of the *franciscana-citrana*
be in close contact in coastal so
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the *franciscana-citrana* complex
whereas *niscana* and a presumably
cies, *lignitaenia,* are restricted to sp
endemic shrubs in the genus *Adeno*
Finally, we discovered coastal
smaller *Argyrotaenia,* here referred
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typical of the *franciscana–citrana*
dark rust-red scaling that character
are closely sympatric with popul
Obispo County interpreted to be
(Powell 1981) and hybrid *francisca*
1965, 1981, and current data), and
evidence of field hybridization. The
entity appear to be specialists on
(based on 6 field collections of larv
and *Lessingia,* J.A.P., unpublished
scription of this new species is plan
pending further morphological exa
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To aid in clarifying relationships
cross-population hybridization tests
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localities. These represent populati
franciscana s. str., *A. f. insulana, A.*
and *Argyrotaenia* n. sp. The metho
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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 phenotypically 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 1965, 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. fransiscana* 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

No.	Taxon	Code(s)	Collecting locality[a] and year, counts
4	*A. citrana*	Acil, 4, 14, 31	Berkeley, Alameda, 1995, 1996
4	*A. citrana*	Aci5, 6, 15, 16	Montana de Oro S.P., San Luis Obispo, 1996
3	*A. citrana*	Aci48[b], 49[b], 50[b]	NAS Miramar, San Diego, 1996
3	*A. citrana*	Aci3, 22[b], 24[b]	Brooks Island, Contra Costa, 1994, 1995
4	*A. citrana*	Aci32, 33, 34, 35	Washington laboratory colony, 1996
1	*A. citrana*	Aci29	Los Angeles, Los Angeles, 1996
4	*A. franciscana*	Af11, 12, 13, 25	UC Bodega Marine Res. Stn, Sonoma, 1996
4	*A. franciscana*	Af18, 19, 20, 21	UC Big Creek Reserve, Monterey, 1996
3	*A. franciscana*	Af39[b], 40[b], 46[b]	Dune Lakes, San Luis Obispo, 1992
3	*A. f. insulana*	Afi2, 8, 17	San Miguel Island, Santa Barbara, 1995, 1996
2	*A. f. insulana*	Afi7, 30	Santa Rosa Island, Santa Barbara, 1995
1	*A. f. insulana*	Afi44[b]	Santa Cruz Island, Santa Barbara, 1984
1	*A. f. insulana*	Afi45[b]	San Nicolas Island, Ventura, 1978
4	A. n. sp.	Ansp9, 10, 42, 43	Montana de Oro S.P., San Luis Obispo, 1996
1	*A. isolatissima*	Aiso52[b]	Santa Barbara I., Santa Barbara, 1986
2	*A. niscana*	Anisc23[b], 38[b]	Santa Rosa Island, Santa Barbara, 1995
2	*A. niscana*	Anisc37[b], 47[b]	Boulder Oaks Campground, San Diego, 1991
1	*A. niscana*	Anisc981	UC Hastings Reserve, Monterey, 1998
1	*A. coloradana*	Acolo26	Arizona, Little Spring, Coconino, 1995
1	*A. klotsi*	Aklotsi27	Arizona, Little Spring, Coconino, 1995

[a] Except otherwise indicated, localities are in California.
[b] Pinned museum specimens.

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

The mtDNA of 2 specimens, from Berkeley (Acil) and San Miguel Island (Afi2), 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 TTT GG 3′ and TL2-N-3013 (Pat2) 5′ TCC ATT ACA TAT AAT CTG 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. klotsi* 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 Acil and Afi2 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 13.0% 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. isolatissima* 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 ata

llecting locality^a and year, counts

ey, Alameda, 1995, 1996
na de Oro S.P., San Luis Obispo, 1996
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odega Marine Res. Stn, Sonoma, 1996
Big Creek Reserve, Monterey, 1996
e Lakes, San Luis Obispo, 1992
Miguel Island, Santa Barbara, 1995, 1996
a Rosa Island, Santa Barbara, 1995
a Cruz Island, Santa Barbara, 1984
Nicolas Island, Ventura, 1978
ntana de Oro S.P., San Luis Obispo, 1996
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       ------ TY-J-1460(K698)--->   .         .         .         .         .     T   .         .         .
1434   tacaatttatcgcctaaacttcagccATTTTATTTAGCGAAAATGACTTTATTCAACAAATCATAAAGATATTGGCACATTATATTTTATTTTTGGAATT
         A      .         .         .         .         .   G     .         .         .         .         .
1540   TGGGCAGGTATAGTAGGAACATCTCTAAGATTATTAATTCGAGCTGAATTAGGAAATCCAGGATCATTAATCGGTGATGATCAAATTTATAATACTATTG
                .         .         .         .   T     .     T  A.         .      G  .         .         .
1640   TAACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCCATTATAATTGGCGGGTTTGGAAATTGATTAGTACCATTAATATTAGGAGCCCCAGA
                .         .    C    .         .         .         .         .         .         .         .
1740   TATAGCTTTCCCCCGAATAAATAATATAAGATTTTGACTTCTTCCACCTTCAATTATACTTTTAATTTCAAGAAGAATTGTAGAAAATGGGGCAGGAACA
                .         .         .  T      . C       .         .         .    T  G .         .        A.
1840   GGATGAACAGTTTACCCCCCTTTATCCTCAAACATTGCTCATAGAGGAAGTTCAGTTGATTTAGCAATTTTTTCCCTACATTTAGCTGGAATTTCTTCGA
                .         .         . T       .         .         .         .         .         .       T .
1940   TTTTAGGTGCAGTAAATTTTATTACAACTATCATTAATATACGACCTAATAATATATCATTAGATCAAATACCTTTATTTGTATGATCTGTAGGAATCAC
                .C        .       C .         .         .         .         .         . T       .C        .
2040   AGCACTTTTATTATTATTATCATTACCTGTATTAGCTGGAGCTATTACAATATTATTAACAGATCGAAATTTAAATACATCCTTTTTCGATCCAGCAGGA
         A      .         .C ----C---(Jerry)----->    T .         T         .         .   A     .     C   .
2140   GGGGGAGACCCTATTTTATATCAACATTTATTTTGATTTTTGGGCACCCAGAAGTTTACATTTTAATTTTACCAGGATTTGGGATAATTTCCCATATTA
                .A        .         .    A  T .         .         .         .         .         .         .
2240   TTTCACAAGAGAGAGGAAAAAAAGAAACTTTTGGGTGCTTAGGAATAATTTATGCTATAATAGCAATTGGTTTACTAGGATTTGTAGTTTGAGCTCATCA
                .         .         .         .        G.         .         .         .         .       C .
2340   TATATTTACTGTAGGAATAGATATTGATACACGAGCTTATTTTACATCAGCAACAATAATTATTGCTGTACCAACAGGTATTAAAATTTTTAGTTGATTA
                . T     C .         .         .         .         .         .         .         .         .
2440   GCAACTTTACACGGAACTCAAATTAATTATAGACCTTCAATACTTTGAAGATTAGGATTTGTATTTTTATTTACTGTAGGAGGATTAACAGGTGTAATTT
                .         .         .         .         .         .         .   C     .         .         .
2540   TAGCTAATTCATCTATTGATGTTACTTTACATGATACATATTATGTTGTTGCTCATTTTCATTATGTACTTTCTATGGGAGCTGTATTTGCAATTATAGG
             C  .         .  T      .         .         .     C   .         .         .       A .         .
2640   AGGATTTGTTCATTGATACCCACTATTTACAGGACTATCAATAAATCCATATTTATTAAAAATTCAATTTTTTACAATATTTATTGGGGTAAATTTAACA
               A.         .         A         .       C .T     T  .         .         .         .         .
2740   TTTTTTCCCCAACATTTTTTAGGTTTAGCGGGAATACCTCGACGATATTCCGATTACCCTGATACATATACTTCATGAAATATTATTTCATCATTAGGAT
                .         .         .         .         .         .         .         .         .         .
2840   CTTATATTTCATTAATTGCAACAATATTAATATTAATTATTGTTTGAGAATCTTTAATTAATAAACGAATTATTTTATTCCCTTTAAACATAAACTCTTC
                .       A .         .         .         .         .A      T   <---------- T--T -(Pat2)--  .
2940   TATTGAATGATATCAAGATCTTCCACCAGCAGAACATTCATATAATGAATTACCTATTTTTAGAAACTTCTAATATGGCAGACTACATGTAATGGATTTA
                .         .         .         .         .         .         .         .        A.         .
3043   AACCCCATTTATAAAGGAATATCCTTTTTTTAGAAATGGCAACATGATCTAATTTTAATCTTCAAAATAGAGCTTCACCTTTAATAGAGCAAATTATTTT
                .         G         .         .         .         .         .         .         .         .
3148   TTTTCATGATCATACTTTAATAATTTTAATTATAATTACTATTTTAGTAGGATACTTAATAATTAGTTTATTTTTTAACTCATATATTAATCGATTTTTA
                .         .         .         .         .         .         .         .         .         .
3248   CTAGAAGGACAAATAATTGAATTAATTTGAACAATTTTACCAGCTATTACATTAATTTTTATTGCATTACCTTCTTTACGACTACTTTATTTATTAGATG
                .T        .         .         .         .         .         .         .         .    T    .
3348   AACTTAATAACCCTTTAATCACATTAAAATCAATTGGTCATCAATGATATTGAAGTTATGAATATTCAGATTTTAATAATATTCAATTTGATTCATATAT
                .         .         .         .         .         .         .A G     C.         .         .
3448   AATCCCTATAAATGAAATAAAAAATGATAATTTTCGATTATTAGATGTTGATAATCGAATTATTTTACCTGTAAATAATCAAATTCGAATTATAGTTACA
                .         .         .         . A       .         .         .         .         .         .
3548   GCTACAGATGTAATTCATTCTTGAACTATCCCCTCCTTAGGTGTAAAAGTAGATGCTAACCCTGGTCGATTAAACCAAACTAATTTTTTTATTAATCGAC
                .      A  .         .         .         .         .      C  .         .         .
3648   CAGGAATTTTTTATGGGCAATGTTCCGAAATTTGTGGTGCTAATCATAGTTTTATACCTATTGTAATTGAAAGAATTTCAATTAAAAATTTTATTAATTG
                .         . <---TK-N-3782(Eva) --------
3748   AATTAATAATTATTCATCATTagatgactgaaagcaagtaatggtctc
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Fig. 1. DNA sequence for *A. citrana* (Berkeley haplotype: Aci1) across mitochondrial COI, tRNA leu, and COII genes. Numbering corresponds to homologous sequence in *D. yakuba* (Clary and Wolstenholme 1985). Sites that differ on haplotype Afi2 (*A. franciscana insulana*, San Miguel Island) are indicated above corresponding bases. Primer locations are indicated above sequence. The Aci1 sequence has been deposited in GenBank under Accession No. AF093681.

specimens of the new species collected at Montana de Oro State Park, San Luis Obispo County.

For mtDNA within the *franciscana* clade (Aci + Af + Afi + Aiso), divergence was up to 3.8% (Aci15 versus Afi45). 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 mtDNAs 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.

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        22222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222
        22222222222222223333333333333333444444444444455555555555555555556666666666666666666677777777777777777788888888888888888899999999999
        23345567777888990111223445677889034455567799901122344556667788990011114455677777888900123444666788899900011223345567889901234555668
        32540621478036581014584695703287676814702816954709247032681769281703690628203479458569274258349214703645814692810687132381849268474
Aci1    GCTAGAATGCTAAATAATTCATTTTATAATAATTTACATAAATATTTAAATTTATTTATATTTTTATTGAATTCCAACTAATATATTGTTTCTTGAAATCTCCATTATAAATATATGTTTAACCATGTAAA
Aci5    .........................G...............T.......................................C.....A...........................................
Aci15   ............G............G...............T.......................................C.....A...........................................
Aci16   .........................G...............T...............................T.......C.....A...........................................
Af39    .........T...............G...............T.......................................C.....A...........................................
Af40    .........................................T.......................................C.....A...........................................
Af12    A...........................G.........C................................C.....T...C............AG..C..T...C..G............G...CA....
Af11    A.....................C.....G.........C................................C.....T...C............A...C..T...C..G............G....A....
Af18    A...A...A.............C.C.............C.........G...................A.GC.........C................C..TT..................G...CA....
Af19    A...A...A.............C.C.............C.........G...................A.GC.........C................C..T...................G...CA....
Aci48   A...A...AT..........................T.C.............................A..C.T.......C.C...A...T..A...C..T...................G....A....
Aci50   A...A...AT..........................TGC.............................A..C.T.......C.C...A...T..A...C..T...................G....A....
Aci3    A...A...AT......G................C..T.C............................CA..C..T........C...A...A..A...CT.T........................A....
Aci29   A...A...AT.......................C..T.C............................CA..C..T........C...A...A..A...CT.T........................A....
Afi2    A.C.A...AT....................G..C..T.C.............................C..C..T........C...A...A..A...CT.T........................A....
Afi17   A.C.A...AT....................G.AC..T.C.............................C..C..T........C...A...A..A...CT.T........................A....
Afi44   ..C.A...AT...G................G..C..T.C.............................C..C..T........C...A...A..A...CT.T........................A....
Afi45   A.TTA.G.ATCC..C...C...................C........................C....A..CCTT........C...A..CA..A...CT.T.....................T..A....
Aiso52  A.T.ATG..TC....G..C......---------------------------..C....C...C....A...CTTG.......C...A...A..A...CT.T..CC....................A....
Ansp9   TATCA...AT....A..C.T.......T.C.G....T.....C..CA.T....TA.....AA...T..A...CTT..T...CT..C.A........G..TCTT....CT..C.CTC.A....T..CA.C..
Ansp42  TATCA...AT....A..C.T.......T.C.G....T.....C..CA.T....TA.....AA...T......CTT..T...CT..C.A...........TCTT....CT..C..TC.A....T..CA.C..
Ansp10  TATCA...AT....A..C.T.......T.C.G....T.....C..CA.T....TA.....AA...T..A...CTT..T...CT..C.A......A....TCTT....CT..C.CTC.A....T..CA.C..
Anis23  TTTCAT..AT....A..C.T......CT........T.....C..CA.T....TA.....AA..CT..A.G...T..T....T...CA..C..CA....T.TT.....T..C..T..AC..GT...A.T..
Anis38  TTTCAT..AT....A..C.T......CT........T.....C..CA.T....TA.....AA..CT..A.G...T..T....T...CA..C...A....T.TT.....T..C..T..AC..GT...A.T..
Anis37  TTTCAT..AT....A..C.T.......T........T..G..CG.CA.T....TA.....AA..CT..A.G...T..T....T...CA..CT..A....T.TT.....T.....T..AC..GT...A.T..
Ani981  TTTCAT...T..G.A..C.T.......T........T.....C..CAGT....TA.....AA..CT..A.G...T..T....T...CA..CT..A....T.TT.....T..C..T..AC..GT...A.T..
Acol26  TTTCATG.ATC........TT.CCA.CT.C..C.CTT.C.....C.AT.TAA.T.CCTC.A.CC.T.AA.....TTCTGTGC.CGCCAC.C.G.A....A...TCAT.TTT.TC..A..C..TTG.A..T.
Aklo27  TTT.A..CA........A.T.AC....T......CTT...TC....AT.....T.ACT.TAAC..TAAAG..CTTT.T....T....T.C....T..G.T...T..TCTTT.....A.....TT..AC..G
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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.

Discussion

The most commonly employed criterion for delineating 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-

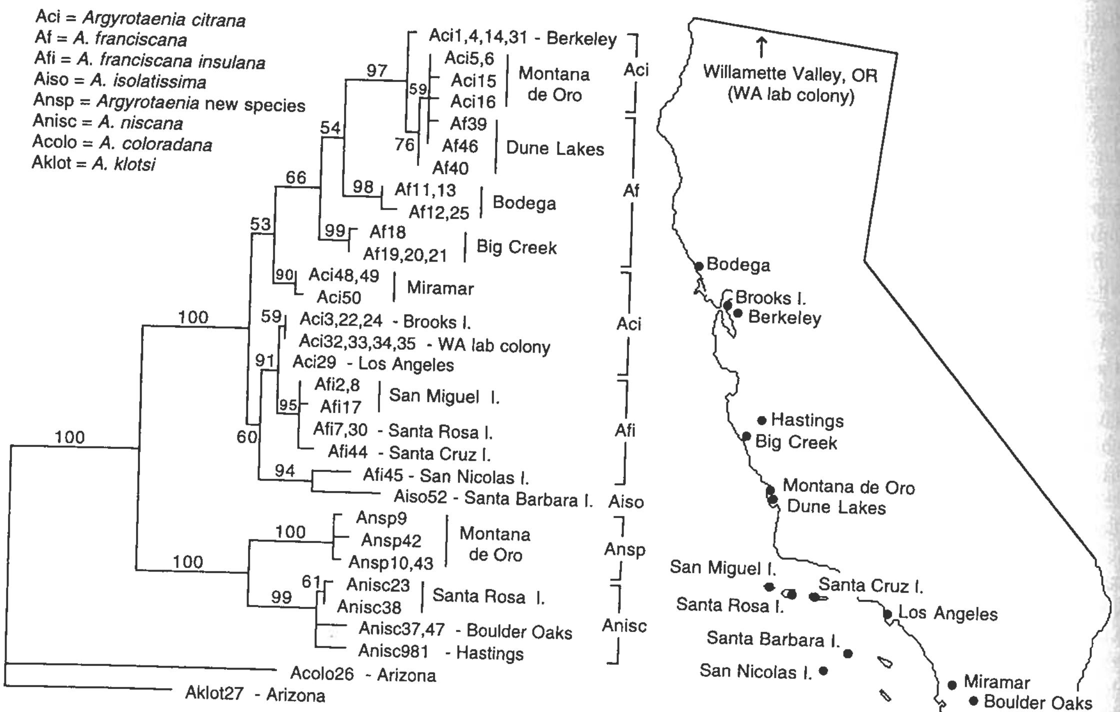

Fig. 3. Phylogram representing relationships of all haplotypes. Numbers above internodes are values of bootstrap support. Locality origins are indicated on a map of California. Specimens with identical haplotypes are shown separately, with zero horizontal branch length between them, when they are found at 2 localities (e.g., Aci5 and Aci6 versus Af46).

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mine whether populations ar
is to use molecular assays to
(Mallet 1995). Our examina
quence variation is a genetic
Although our study focuses
relatively unusual maternal i
the advantage of being pote
genetic bottlenecks (Moore
best single indicator of speci
(Sperling 1994). Furthermor
of heteroplasmy, or mtDN
within individuals, and the r
vergences between species a
phylogenetic inference. The
lend themselves to interpret
netic species concept that v
detectable monophyletic pop
Wheeler 1990).
Application of the phyloge
Argyrotaenia mtDNA suggest
citrana, franciscana, f. insulan
latissima, form 1 species. This
A. franciscana, because it is th
Sequence divergence withi
mtDNA lineage is relatively
quence divergence within thi
higher than the maximal perce
between *A. niscana* and the
higher than results obtained i
Lepidoptera. In a study of th
region in pheromone types
(Noctuidae), maximal seque
species was 2.3%, and 3.7% w
to belong to a sibling species
ling et al. 1996). Among sub
looper (Geometridae), 2.2%
curred in a larger segment h
sequenced in single specime
franciscana, which had a diver
et al. 1999). Within the *C. fum*
species, Tortricidae), 2.9% di
1.6-kb fragment of COI an
Hickey 1994). Finally, amon
moths (Yponomeutidae) <1
across the 2.3 kb COI+II regi
Thus, simple percent sequen
closely related sister species
variable and is not necessari
whether 2 unknown populatio
tively isolated species. How
mtDNA divergences within *A.*
deep and presumably old.
The lack of support for mon
A. franciscana or *A. citrana* is
vations of phenotypic change
Francisco Bay area and Santa
gests hybridization in distu
1964, 1965, and unpublished da
laboratory hybridization trial
data).
Based on our data, *A. franc*
isolatissima probably should al

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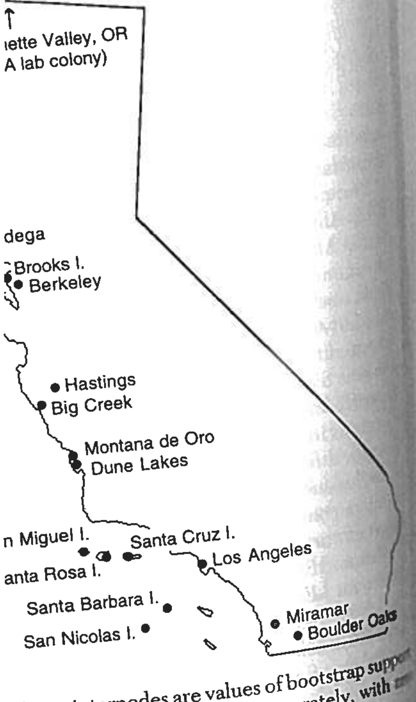
2222222222222222222222222222222222222
7777777788888888888888888899999999999
5788899900011223345567889901234555668
9214703645814692810687132381849268474
GAAATCTCCATTATAAATATATGTTTAACCATGTAAA
.....................................
.....................................
.....................................
.....................................
..........................G....CA....
.AG..C..T...C...G.........G.....A....
.A...C..T...C...G.........G....CA....
......C..TT...............G....CA....
......C..T................G.....A....
..A...C..T................G.....A....
..A...C..T......................A....
...A...CT.T.....................A....
A..A...CT.T.....................A....
A..A...CT.T.....................A....
A..A...CT.T.....................A....
A..A...CT.T.................T...A....
A..A...CT.T.....................A....
A..A...CT.T..CC.............T..CA.C..
......G..TCTT....CT..C.CTC.A....T..CA.C..
.........TCTT....CT.:C..TC.A....T..CA.C..
....A....TCTT....CT..C.CTC.A....T..CA.C..
C..CA....T.TT....T..C..T..AC..GT...A.T..
C...A....T.TT....T..C..T..AC..GT...A.T..
.CT..A....T.TT....T.....T..AC..GT...A.T..
.CT..A....T.TT....T..C..T..AC..GT...A.T..
.C.G.A....A...TCAT.TTT.TC..A..C..TTG.A..T.
C....T..G.T...T..TCTTT.....A.....TT..AC..G
```

Dashes in Aiso52 indicate where no

ole amount of postzygotic isolation ied data). However, laboratory hybe relatively uninformative about ng mechanisms that operate only unitions. An alternate means to determine whether populations are reproductively 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 heteroplasmy, 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).



s above internodes are values of bootstrap supp... with ... ntical haplotypes are shown separately, with ... ties (e.g., Aci5 and Aci6 versus Af46).

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 sequenced in single specimens of *A. citrana* 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 reproductively 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*.

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Temporal Pattern
Phyllophaga (Col

ROBERT L. CRO

Texas Agricultural Experiment

ABSTRACT The fecun
chard), *Phyllophaga rubi*
abaeidae) were measure
beetles from samples coll
TX. *Phyllophaga hirtiven*
followed by *P. rubiginosa*
female; n = 643), and *P.*
female decreased linearl
progressed. Egg counts fr
reproductively immature
27.3% of *P. rubiginosa* fem
contained no mature egg
counts in *P. congrua* gav
percentage frequency of
negative binomial distrib
counts. *Phyllophaga crass*
times as many even-numb
indicated that the distrib
than was that of *P. crassiss*
was least in *P. congrua* (

KEY WORDS *Phylloph*

Entomologists long have been i
production of scarabaeids and ot
for understanding the ecology a
species. Davis (1916) reported t
lophaga (*Lachnosterna*) spp. femal
average of >50 (maximum of ≥10
then, the recovery of eggs deposit
has been widely used to estimate
of diverse scarabaeids (Baerg 19
inhard 1942, Tashiro et al. 1969, Fl
et al. 1979).

Four species of *Phyllophaga*
(UV) light insect traps in the sprin
Co., TX. Of these spring species (
to collectively as "May beetles"),
(LeConte) is the most abundant,
sissima (Blanchard), *P. rubiginos*
few *P. hirtiventris* (Horn) (R.L.C.,
All 4 of these beetles normally hav

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