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DEFINING *CLEPSIS PENETRALIS* RAZOWSKI (TORTRICIDAE) USING MORPHOLOGY AND MOLECULES: A WIDESPREAD BUT OVERLOOKED NORTH AMERICAN SPECIES

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**ABSTRACT.** The taxonomic status of *Clepsis penetralis* has remained enigmatic since its description in 1979. Using specimens collected or borrowed from across the U.S.A., we examined genitalic and wing characters as well as mitochondrial DNA sequence in order to distinguish *C. penetralis* from the similar congener *C. peritana*. The genomic integrity of the two species was strongly supported, and the mtDNA sequence data further suggest a potential additional new species from California. Examinations of collections across the country indicate that *C. penetralis* is a widespread species that has been widely overlooked.

**Additional key words:** Tortricinae, Archipini, Cytochrome Oxidase Subunit I (COI), mtDNA, systematics

In his comprehensive taxonomic revision of the genus *Clepsis* (Guenée), Razowski (1979) described *C. penetralis* from three localities in Utah. The status of this species has remained uncertain because the characters that best define it were lost from the only available female specimen, and because Razowski's illustration of them is unverifiable. He assigned *C. penetralis* to his Subgroup 1, which is defined primarily by having a "normally" developed ductus bursae (i.e. not spiralled and without a cestum).

Razowski compared the new species with *C. smicrotes* (Wlsm.), which was described from Guerrero, Mexico, but *C. penetralis* phenotypically more closely resembles the sympatric, widespread *C. peritana* (Clemens). The latter is a member of Razowski's Subgroup 2, species having a tightly coiled ductus bursae with a weakly sclerotized cestum along its length (Powell 1964, fig. 104; Razowski 1979, figs. 77–90). The female of *C. penetralis* illustrated by Razowski has the ductus bursae simple and uncoiled, lacking a cestum, and the corpus bursae lacking a signum.

A third species, *C. virescana* (Clemens), which is broadly sympatric in North America, is similar in forewing pattern but differs from the other two in possession of a costal fold in the male and by possession of an elongate, sclerotized antrum and well developed signum in the female (Powell 1964, fig. 100).

Razowski (1979) cited four males of *Clepsis penetralis*: the holotype from Logan, Cache Co., and three paratypes from Hooper, Weber Co. (for which four dates are listed), and one female from Johnson Pass, west of Clover, Tooele Co. Hence, association of

the sexes was equivocal. Unfortunately, the slide containing the female dissection has only the external sclerotized structures and a damaged abdominal pelt; examination under high magnification reveals no trace of the bursa copulatrix. As a result, the accuracy of the illustration cannot be verified, and we have been unable to locate another specimen possessing the peculiar combination of characters: forewing pattern with the dark markings and ground color resembling *C. peritana*, and the ductus bursae simple, gradually enlarged distally, resembling that of *C. virescana* but without the elongate antrum of that species.

In 1992 JAP obtained additional specimens that match the holotype of *C. penetralis* from Garfield Co. in southern Utah. Males and females are similar in forewing size, breadth, and color pattern, and this series has enabled definitive recognition of the species.

## MATERIALS AND METHODS

**Specimens.** The specimens used in this study were provided by collaborators or were collected by the authors. The two outgroups were selected from related Archipine genera. Outgroup taxa from related genera were used from previously published work: *Argyrotaenia niscana* Kearfott from Santa Barbara County, CA (Landry et al. 1999); and *Choristoneura rosaceana* Harris from Ste. Agathe, Quebec (Sperling & Hickey 1994). Outgroups were chosen on the basis of presumed distant relationship but within the same tribe.

We attempted to obtain specimens from a diversity of sites across the ranges of ingroup species. Where possible, we sampled at least two specimens of each

species from each location to determine the extent of sequence divergence and to test species concepts. Specimens were collected using lights (ultraviolet, mercury vapor, or incandescent), searching foliage, or rearing from larvae collected in the field. Representative specimens were photographed using a Leica M16 Zoom Stereomicroscope with a Leica DSC 320 3MP digital camera using a DFC Twain 6.6.1 (2006) driver for Windows at the University of Alaska Museum. Images (Figs. 1–4) were manipulated in Photoshop. For molecular analyses, live specimens were either frozen at  $-20^{\circ}\text{C}$ ,  $-70^{\circ}\text{C}$ , or dropped directly into 95–100% EtOH. Pinned museum specimens were used for the morphological portion of this study and to supplement the fresh specimens in the molecular portion when possible.

Specimens were identified initially by phenotype, specifically forewing pattern, prior to DNA extraction or dissection for slide preparation. The unused body parts of each specimen were preserved in a gelatin capsule for confirmation of identification, and these vouchers are deposited in the Essig Museum of Entomology (EME) or the National Museum of Natural History (NMNH).

**Specimens examined of *Clepsis penetralis* Razowski (11 m, 9 f):** CALIFORNIA: Whitney Trail (1 ♂), Tom's Place (1 ♀), Berkeley (1 ♀). COLORADO: Fort Collins (1 ♂). CONNECTICUT: Hampton (1 ♂). UTAH: Hooper (3 ♂), Bryce Jct. (3 ♂, 4 ♀), Ogden (1 ♂), Johnson Pass (1 ♀). VERMONT: Burlington (2 ♀). WASHINGTON: Brewster (1 ♂).

**Specimens examined of *Clepsis peritana* (Clemens) (32 m, 22 f):** ALASKA: Cantwell (1 ♂). CALIFORNIA: Albany (4 ♂), Bakersfield (3 ♂, 1 ♀), Berkeley (2 ♂, 8 ♀), Davis (2 ♂), Herbert Creek (1 ♀), Mission Gorge (1 ♀), Orinda (1 ♂), Pleasant Hill (2 ♂, 2 ♀), Richmond (1 ♂), San Lorenzo (4 ♂), Santa Cruz Island (1 ♀), Shafter (1 ♂), Walnut Creek (1 ♂, 1 ♀). CONNECTICUT: Hampton (4 ♂). MASSACHUSETTS: Sturbridge (1 ♀). MARYLAND: Laurel (2 ♂). MICHIGAN: no further info (1 ♀). TENNESSEE: Crosby (1 ♀). UTAH: Springdale (2 ♂). VIRGINIA: Alexandria (3 ♀). WISCONSIN: Lake Katherine (2 ♂, 1 ♀).

Eight specimens were selected for the molecular portion of this study: two *Clepsis penetralis* from Garfield Co., UT; two *C. peritana* from Berkeley, CA; three *C. peritana* from the Rutherford neighborhood, east of Fairfax City, VA, and one *C. peritana* from Putnam Co., IL.

**Morphological techniques.** Dissection methods follow those summarized in Brown & Powell (1991). Terminology for genital structures follows Horak (1984). Eighteen *C. penetralis* and ten *C. peritana* were also physically measured for forewing (FW) length and

FW width. Measurements were plotted on a scattergram (Fig. 5).

**Molecular techniques.** Total genomic DNA was extracted using a QIAamp DNA Mini Kit # 51306 (QIAGEN Inc., Valencia, CA, U.S.A.). Most amplified fragments were approximately 400–500 basepairs long. Amplifications were performed on an Ericomp TwinBlock EasyCycler using a hot start: Taq was added at the end of an initial denaturation at  $94^{\circ}\text{C}$ , followed by 35 cycles of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $45^{\circ}\text{C}$ , 1 min at  $72^{\circ}\text{C}$ , and a subsequent 10 minute final extension at  $72^{\circ}\text{C}$ . For many of the older museum specimens, amplifications were performed on an MJ Research PTC200 using a hot start: Taq was added at the end of an initial denaturation at  $94^{\circ}\text{C}$ , followed by 10 repetitions of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $40^{\circ}\text{C}$  and 40 s at  $72^{\circ}\text{C}$ , 10 repetitions of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $45^{\circ}\text{C}$  and 40 s at  $72^{\circ}\text{C}$ , and 15 repetitions of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $50^{\circ}\text{C}$  and 40 s at  $72^{\circ}\text{C}$ , and a subsequent 3-minute final extension.

PCR products were cleaned using a QIAquick PCR Purification Kit #28106 (QIAGEN Inc.). The PCR product was cycle sequenced with a Perkin-Elmer/ABI Dye Terminator Cycle Sequencing Kit with AmpliTaq FS (Perkin-Elmer/Applied Biosystems, Foster City, CA, U.S.A.) on an MJ Research PTC200 according to Perkin-Elmer's suggested thermal profile. The sequenced product was filtered through Sephadex-packed columns and dried. This product was resuspended and electrophoresed on an Applied Biosystems International 377 automated sequencer. All fragments were sequenced in both directions. Sequences were aligned manually to the sequence of *Drosophila yakuba* Burla (Clary & Wolstenholme 1985).

We chose an 816 basepair segment in the COI gene to compare 8 specimens from 2 species of *Clepsis*, and 1 specimen of each of the 2 outgroup species. This fragment corresponds to the second half of COI, between *Drosophila yakuba* basepair numbers 2184 and 3000. Sequence was obtained by PCR amplification using the end primers CI-J-2183: 5' CAA CAT TTA TTT TGA TTT TTT GG 3', CI-N-2659: 5' GAT AAT CCT GTA AAT AAA GG 3' and TL2-N-3014: 5' TCC AAT GCA CTA ATC TGC CAT ATT A 3'. *Choristoneura rosaceana* and *Argyrotaenia niscana* sequences are from other studies (Sperling & Hickey 1994; Landry et al. 1999).

**Phylogenetic analyses.** Analyses using parsimony were carried out using PAUP 4.0b10 (Swofford 2002). Sequence alignments were done manually, and no indels were found relative to *Drosophila yakuba*. Variable nucleotide positions and morphological characters were treated as unordered characters with one state for each nucleotide or character. We employed

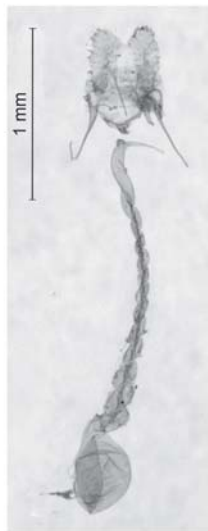




1.



2.



3.



4.

FIGS. 1–4. 1, Wing pattern photograph of *Clepsis peritana* (UT: Springdale, 19–20.VII.1993, J.A. Powell). 2, Wing pattern photograph of *Clepsis penetralis* (VT: Burlington, 25.VI.1987, D.L. Wagner). 3, Female genitalia photograph of *C. peritana* (VA: Alexandria, 17.VII.1991, slide JAP7329). Note the tightly twisted ductus bursae. 4, Female genitalia photograph of *C. penetralis* (UT: Bryce Jct. 28.VIII.1996, slide JAP7357). Note the slender, weakly twisted ductus bursae with faint cestral sclerotization.

some blackish or dark brown suffusion in the median transverse band and subapical costal spot, which usually are strongly contrasting with the tan ground color, appearing dark chocolate brown to the unaided eye (Fig. 1). In *C. penetralis* the forewing ground color tends to be paler, more yellowish tan; the markings are usually pale reddish brown, and if tinged with darker scaling, this tends to be restricted to the costal margin (Fig. 2).

Females of *C. penetralis* possess a slender weakly twisted ductus bursae with faint cestral sclerotization

(Fig. 4). If the original female was correctly associated, the illustration of the ductus bursae was poorly rendered or represents an attempt to reconstruct severely damaged structures that were lost prior to mounting. *Clepsis peritana* differs markedly, having a tightly coiled ductus bursae with central sclerotization (Fig. 3).

Differences in male genitalia between *C. peritana* and *C. penetralis* are subtle: the aedeagus is unevenly bent distally in *C. peritana* (Powell 1964, fig. 53) while it is evenly tapered in *C. penetralis* (Razowski 1979, fig.

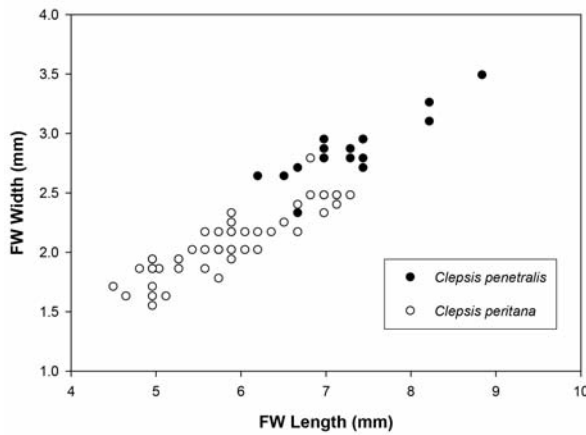


FIG. 5. Scattergram comparing forewing lengths and widths of adult male and female *Clepsis penetralis* [n=18] and *C. peritana* [n=49].

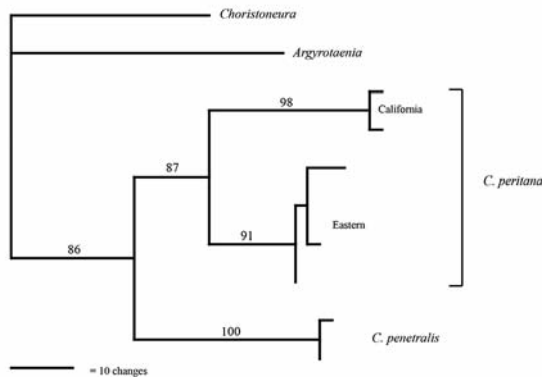


FIG. 6. Representative phylogram from five most parsimonious unrooted trees resulting from heuristic search of 74 molecular parsimony-informative characters from 8 ingroup mtDNA COI haplotypes (161 steps, CI = 0.876; RI = 0.851; RC = 0.745). Numbers at branch nodes indicate bootstrap values from the best Maximum Likelihood tree using GARLI 2.0 and Sumtrees 3.1.0. Only bootstrap values > 50% are shown.

23). Razowski also described two thin cornuti in the vesica of *C. penetralis*, but these have not been found in several dissections, so they may be deciduous. Razowski examined just one dissection of *C. penetralis*, from a paratype that was not returned to EME. We examined specimens mixed with *C. peritana* in the Essig Museum and University of Connecticut collections and discovered several additional females having the weakly coiled ductus bursae.

Adults of *C. penetralis* have forewing length of 6.2–8.8 mm, mean 7.5 mm [n=9] in males and 6.5–7.4 mm, avg. 7.0 mm [n=9] in females and are larger than those of *C. peritana* (FW length males: 4.5–6.4 mm, avg. 5.5 mm [n=27]; females 5.0–7.3 mm, avg. 6.4 mm [n=21]). *Clepsis penetralis* possesses a slightly broader forewing (avg. 1 mm broader), irrespective of sexual dimorphism in forewing length within and between populations (Fig. 5).

DISCUSSION

The utility of mitochondrial DNA sequence analyses in systematic studies at the species level has been demonstrated previously in studies involving the family Tortricidae (Sperling & Hickey 1994, Newcomb & Gleeson 1998, Landry et al. 1999, Kruse 2000, Kruse & Sperling 2001, 2002). Mitochondrial genes provide a wealth of variation that may be particularly useful in generating phylogenetic trees of taxa where morphological differences are subtle (Sperling & Hickey 1994, Cognato et al. 1999, Kruse & Sperling 2001). However, mitochondrial DNA by itself represents only one linked, maternally inherited gene system, and may legitimately have different phylogenies or patterns of variation as the rest of the genome (Sperling & Roe 2009). Combined data sets that involve molecular, morphological, and/or ecological data in insects have led to better and more resolved knowledge of systematic relationships than have analyses of any single data set alone (Miller et al. 1997, Damgaard et al. 2000, Normark 2000, Skevington & Yeates 2000, Kruse & Sperling 2002).

In this study, the integrity of *C. penetralis* and *C. peritana* species concepts was strongly supported in morphological and molecular analyses. Support was found in molecular analyses to potentially describe a new species of *Clepsis* from California (specimens from TX and FL also seem to cluster with those from California, according to the BOLD database). Examinations of collections across the country indicate that *C. penetralis* is a widespread species that has been widely overlooked. According to our data, *Clepsis peritana* from Alaska tend to be as large as *C. penetralis* (male FW length 6.8 mm; n=1). A single female *C. peritana* from Michigan was also measured as quite large (FW length 7.1 mm; n=1). Therefore, confirmation via genitalic dissection is still recommended as some populations and/or some individuals are as large as *C. penetralis*.

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