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Source: The Journal of the Lepidopterists' Society, 67(4) : 253-262

Published By: The Lepidopterists' Society

URL: https://doi.org/10.18473/lepi.v67i4.a2

Journal of the Lepidopterists' Society
67(4), 2013, 253–262

DELIMITATION OF *PHANETA TARANDANA* (MÖSCHLER 1874) AND *P. MONTANANA* (WALSINGHAM 1884) (TORTRICIDAE: OLETHREUTINAE) IN WESTERN CANADA USING MORPHOLOGY AND DNA

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ABSTRACT. *Phaneta* comprises a large genus of Nearctic tortricids with several species that are difficult to delimit using morphological characters. We examined *Phaneta tarandana* (Möschler 1874) and *P. montanana* (Walsingham 1884), using morphology and DNA. Our results upheld the distinction between these two species, even though the wing coloration and male genital characters traditionally used to diagnose them were unreliable due to intraspecific variation. Nonetheless, a combination of forewing fringe scale detail, wing streaks, mitochondrial, and nuclear DNA delimited these moth species in a region of overlap in western Canada.

Additional key words: ITS2, COI, wing fringe, cryptic species

Phaneta Stephens was proposed as a genus in 1852 and the delimitation of its species has challenged taxonomists ever since. In North America, *Phaneta* is a speciose group closely related to *Eucosma*, separable only by the absence of a costal fold in males, a distinction of convenience with no phylogenetic justification (Heinrich 1923; Razowski 2003; Gilligan et al. 2). Currently 260 species of these two genera are recognized in North America, of which 39 *Phaneta* are found in Alberta, Canada (Pohl et al. 2010; Gilligan et al. 2008). Two *Artemisia*-feeding species (Robinson et al. 2002; Brown et al. 2008), *Phaneta tarandana* (Möschler 1874) and *Phaneta montanana* (Walsingham 1884), provide an example of the taxonomic difficulties in the genus. They are part of a species complex that may include other *Phaneta* in the western and south-central United States, such as *Phaneta transversa* (Walsingham 1895), *Phaneta benjamini* (Heinrich 1923), and *Phaneta clarkei* Blanchard & Knudson (1983).

Möschler (1874) described *P. tarandana* based on 3 males from Labrador. The fate of those specimens is unknown. Heinrich (1923) suggested that the "type" might be a specimen from the Fernald Collection, presently in the USNM, that was collected in Labrador and "bears Möschler's label". However Brown (2005) reported the *P. tarandana* holotype as lost. Möschler's description relied on forewing characters but not genitalia. He distinguished this species from other *Phaneta* through its "noticeable size", recording its wingspan as 25-27 mm (Möschler 1874). Walsingham (1884) based his description of *P. montanana* on the forewing characters of one male specimen from Montana. *Phaneta montanana* and *P. tarandana* have not been rigorously reevaluated since their original descriptions.

Recent collections from western Canada have produced an abundance of material putatively representing *P. montanana*, *P. tarandana*, and what appear to be various intermediate forms. This region constitutes a potential zone of interaction between montane and boreal species, whether by overlap or hybridization (e.g. Lumley and Sperling 2010). In this study, we reexamine these two putative *Phaneta* moth species using multiple character types, with the aim of determining how many species they represent and how they may be identified.

MATERIALS AND METHODS

Samples and species criteria. We examined 130 specimens, mainly from Alberta but ranging from Kamloops BC to Prince Albert SK (locations mapped in Fig. 1). Wing pattern, color, and size were examined in all specimens, while a subset of specimens was sequenced for one mitochondrial and one nuclear gene. Outgroups for molecular analyses were selected from GenBank sequence data available for other *Phaneta* species. The species status of *P. montanana* and *P.*

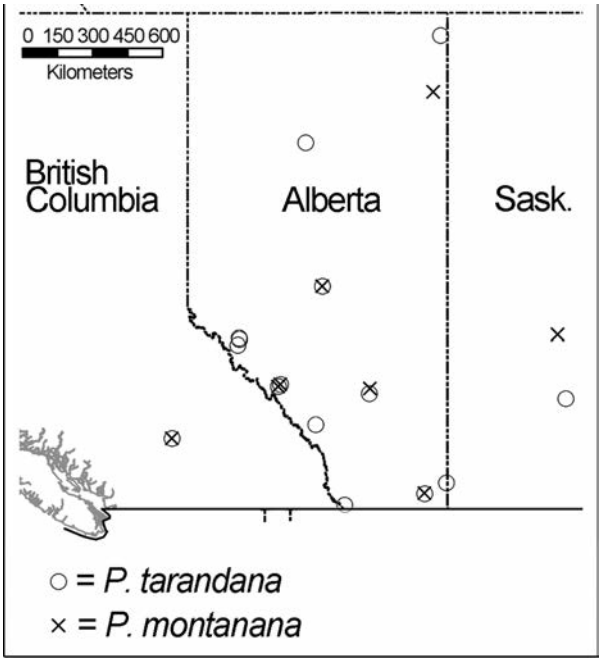

FIG. 1. Geographic locations of sample sites for *P. tarandana* (open circles) and *P. montanana* (crosses) in western Canada. Identifications are based on DNA sequences, forewing ground color, wing fringe and wing streaks, as described in the text.

tarandana was examined under the genomic integrity (Sperling 2003) and general lineage concepts (De Queiroz 1998, e.g. Dombroskie and Sperling 2012), which emphasize the use of multiple stable and consistent criteria to evaluate the distinctness of populations.

Morphology. Wing character terms used here are consistent with those of Razowski (2003). For wing morphometric analyses, the angles of the tornus and apex of the forewing were measured in the same specimens used in molecular analyses (12 male *P. montanana* and 25 *P. tarandana*), using digital images and the measure tool in Photoshop CS4 version 11.0.2 (Adobe Systems Incorporated, CA, USA). Other wing characters examined included ground color, fascia color, longitudinal wing streaks, and coloration of individual scales (See Fig. 2) of the forewing fringe (see Fig. 3 for general wing coloration, and Fig. 4 for forewing fringe scale character states). Ground color is that of the inter-fascia regions and strigulae. We define fascia color as the color present in the dorso-postbasal blotch, median fascia, terminal fascia, and divisions between strigulae.

Ground color and fascia color were examined because they traditionally have been used to delimit tortricid species (e.g. Heinrich 1923). Wing streaks were chosen because we found the character to be variable between specimens and clearly present in the adult drawing of Walsingham's illustration of the holotype of *P. montanana*, but lacking in the description of *P. tarandana*. Patterning on the forewing fringe scales was chosen because it was observed to be variable and is a non-traditional character for which taxonomic utility had not been previously evaluated in *Phaneta*. Heinrich (1923) used forewing fringe colouration to delimit *P. tarandana* from *P. transversa*; however the taxonomic utility of this character has not been tested since Heinrich (1923). We describe and illustrate this character here due to our resurrection of forewing fringe coloration for species delimitation. Wing characters were examined in all specimens, but were only scored in specimens with genetic information. Pearson's Chi-squared test (Naiman et al. 1972) was used to evaluate the statistical significance of associations between wing characters and DNA sequences.

Dissection methods follow Brown et al. (2009) except as follows. Dissections were performed under an M5-72711 Wild dissecting microscope. Genitalia were stained with chlorozol black. Female genitalia were removed using iris scissors to cut the intersegmental membrane between the 7th and 8th abdominal segments. Once open, the genitalia were placed on a pre-cleaned Gold Seal® microscope slide No. 3010, then one drop of glycerol was placed above the genitalia and a square glass cover slip was placed over the genitalia. Genitalia were photographed using a Nikon Coolpix 8400 attached to an Olympus S2X16 microscope, with illumination provided by an Olympus LG-PS3 fiber optic light source. After photography, genitalia were placed in vials along with the abdomen and pinned with the specimen. Genitalic terminology follows Gilligan et al. (2008).

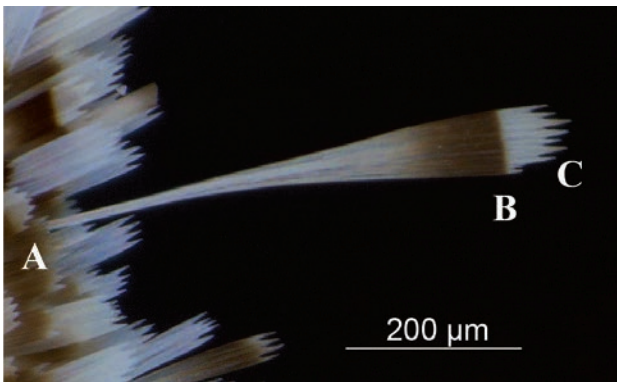

FIG 2. Individual scale along the termen of a *Phaneta tarandana* showing the root (**a**) and gradient origin (**b**) at the base of the apical teeth (c).

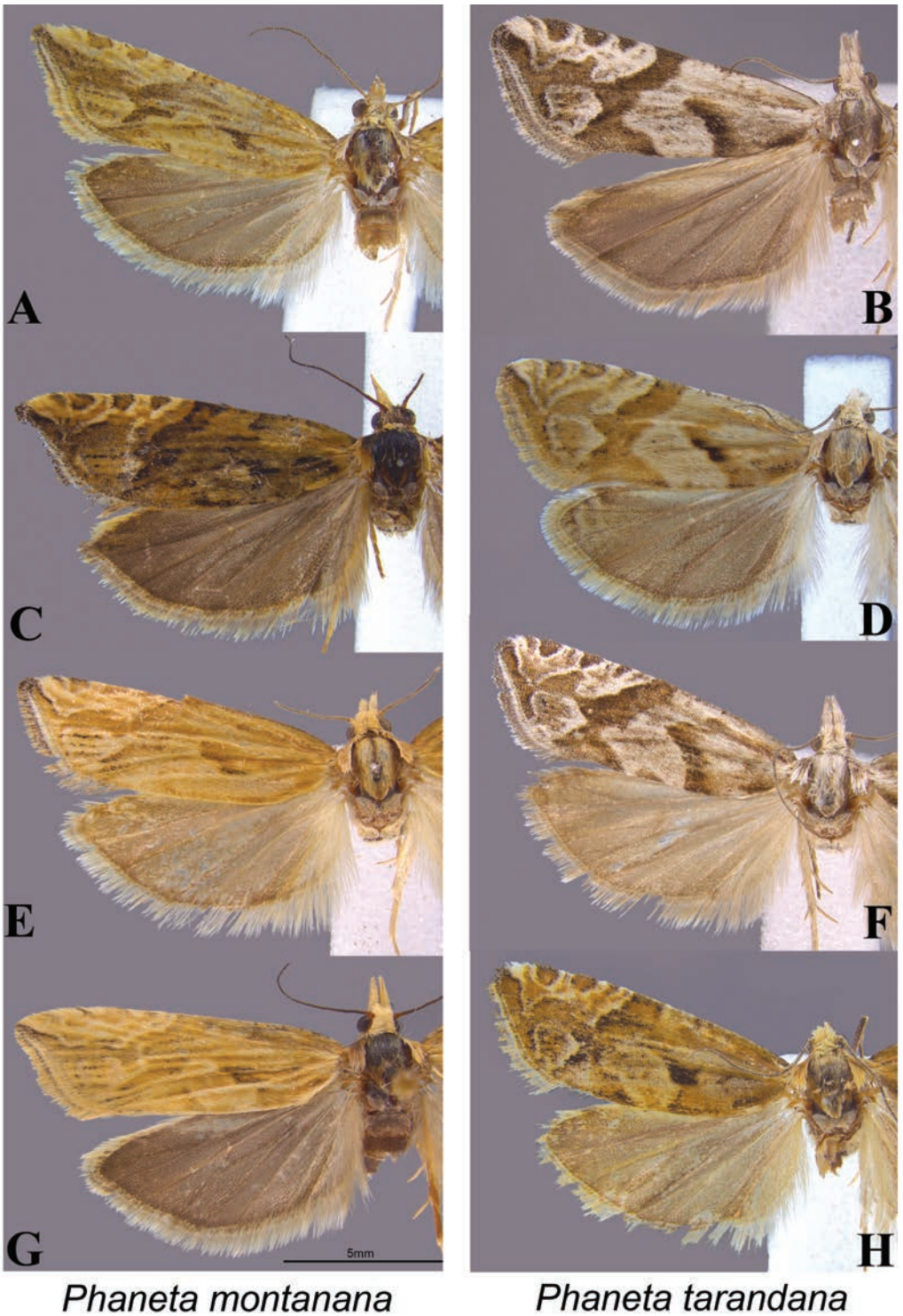

FIG 3. Wing coloration variation of *P. montanana* (right) and *P. tarandana* (left)

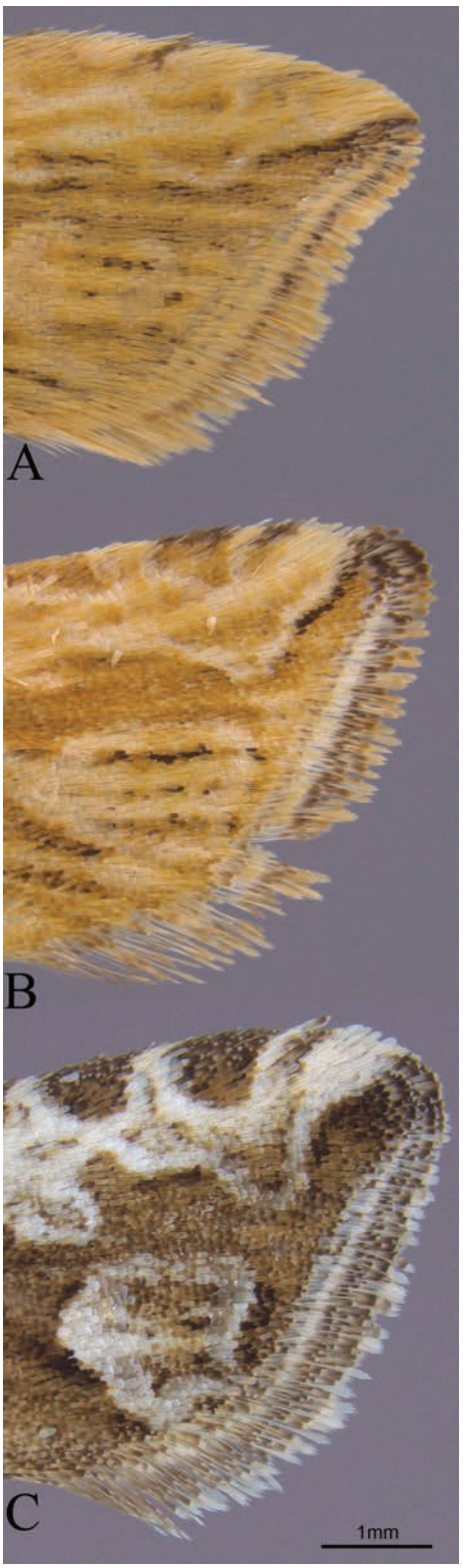

FIG 4. Forewing fringe of a typical *P. montanana* (**a**), *P. montanana* with darker scales (**b**) and typical *P. tarandana* (**c**).

Selection of samples for DNA analysis. Specimens were chosen for DNA analyses to represent a wide range of specimen age and wing coloration. We selected recently collected moths that varied in wing pattern from apparently typical (i.e. matching the species description) *P. tarandana* to typical *P. montanana* as well as intermediates and strongly marked specimens agreeing with the description of *P. transversa* (Walsingham 1895). The main wing character used to choose specimens was streaked coloration on the forewings (Fig. 3). Typical *P. montanana* exhibit strong streaks from the base of the wing to the apex, while *P. tarandana*, according to the original description, has distinct fasciate markings that are not smeared across the wing. The only characters that distinguish *P. transversa* from *P. tarandana*, based on Walsingham's (1895) description, are the presence of more distinct markings in *P. transversa* and a darker patch at the base of the forewing.

DNA extraction, amplification and sequencing. We removed 3 legs from the left side of each specimen; legs were stored in 95% ethanol at -20°C until DNA was extracted. Extractions followed the QIAamp Mini Kit (Qiagen, Canada) protocol with a modification of the final elution steps. To further concentrate DNA we eluted 50µl three times to a final volume of 150µl. PCR protocols followed Lumley & Sperling (2010) for COI and Dombroskie and Sperling (2012) for ITS2. Primers for COI were LCO (GGTCAACAAATCATAAAGATATTGG) and HCO (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al. 1994); those for ITS2 were ITS2F (TGTGAACTGCAGGACACATGAA) and ITS2R (ATGCTTAAATTTAGGGGGTAGTC) (White et al. 1990). PCR product was purified using ExoSAP-IT (USB corporation, Cleveland, OH). BigDye Terminator version 3.1 cycle sequencing (Applied Biosystems, Foster City, CA) was used for the sequencing reaction. Dye-labeled DNA was further purified using ethanol precipitation, then run on an ABI Prism 3730 DNA analyzer (Molecular Biology Service Unit, University of Alberta, Canada).

Analysis of DNA sequences. SeqMan Pro version 7.2.0 (DNASTAR) was used to examine chromatograms and assemble contigs; sequences were then aligned manually using Mesquite version 2.73 (Maddison & Maddison 2010). Parsimony and bootstrap analyses were performed using PAUP 4.0 (Swofford 2003) with 1000 replicates each. Likelihood bootstrapping (100 replicates) used Garli version 2.0 (Zwickl 2006) under settings consistent with the GTR + I + G model, as determined by Modeltest (Posada & Crandell 1998). Posterior probabilities were calculated using Bayesian analyses in MrBayes v3.1.2 (Ronquist & Huelsenbeck

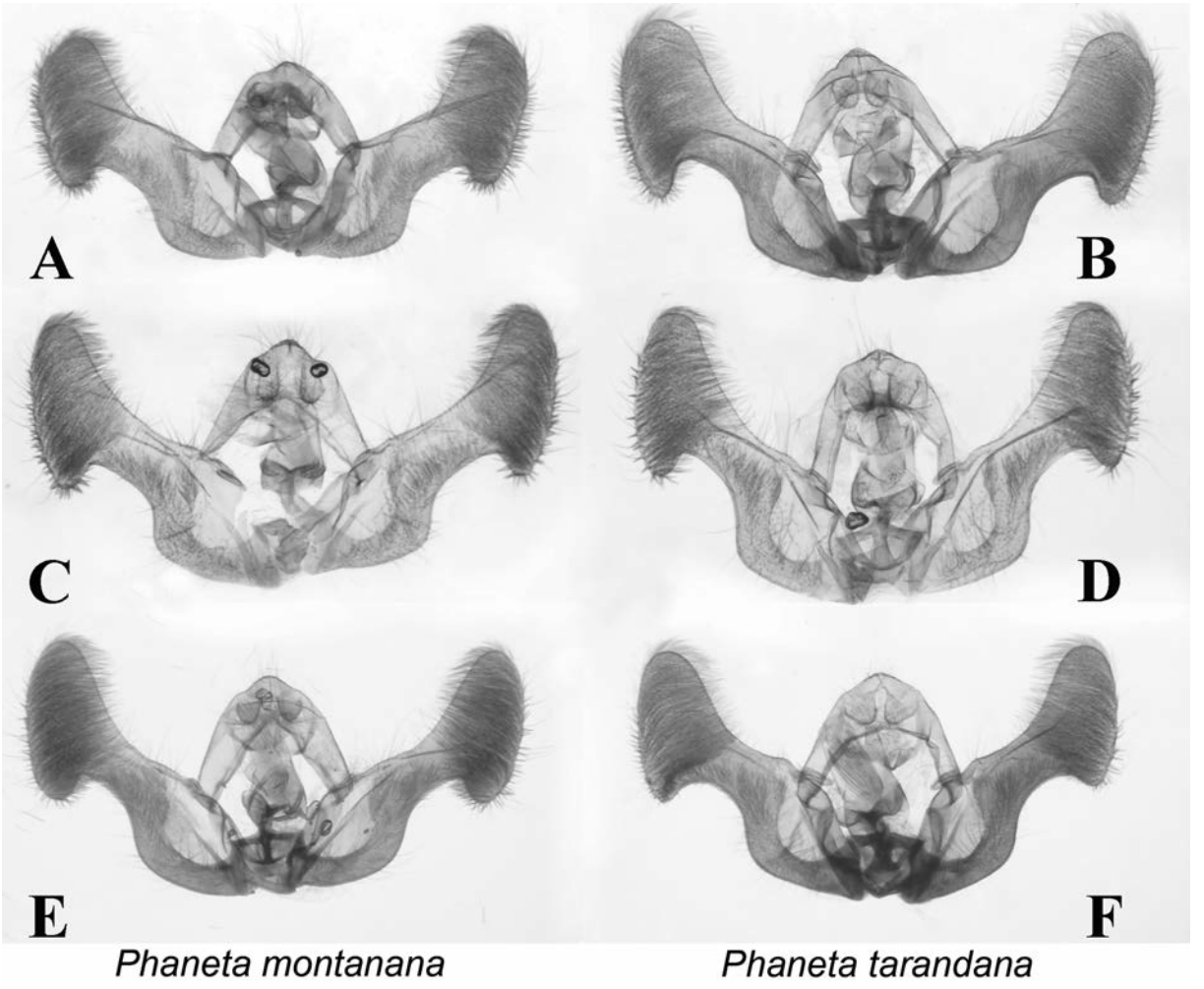

FIG. 5. Male genitalia of representative specimens of *P. tarandana* (right) and *P. montanana* (left).

2003) with default settings and two sets of 120,000 generations. Outgroups for COI were downloaded from GenBank as follows: *P. striatana* (Clemens 1860), GU088462.1; *P. elongana* (Walsingham 1879), HM863962.1; *P. corculana* (Zeller 1874); HQ683323.2, and *P. autumnana* (McDunnough 1942), HQ964424.1.

RESULTS

Morphological variation. For the following, descriptors for individual scales include "root", referring to the tapered base of each scale that attaches to the wing membrane, and "apical teeth", referring to the widened jagged edge of the distal portion of scales. For wing character variation, we identified two character states for fascia color: (1) dark brown to grayish brown (Fig. 3 b,c,f), and (2) yellowish tan to light brown (Fig. 3 a,d,e,g); three character states for ground color (interfascial coloration): (1) yellowish to light tan (Fig. 3 a,d,e,g,h), (2) white (Fig. 3 b,f), and (3) brown (Fig. 3 c); and three for wing streaks: (1) wing streaks strongly present (Fig. 3 a,c,e,g), (2) absent (Fig. 3 b,d) and (3) faintly present (Fig. 3 f,h). Wing fringe scale coloration exhibited three character states: (1) wing fringe scales on termen with a distinct dark band (median band) below the apical teeth fading into the root (Fig. 4 c, also present in Fig. 3 b,c,d,f,h); (2) Cilia concolorous throughout, amelanistic, therefore lacking gradient entirely; and (3) dark median band present on wing fringe scales, but without a distinct origin of the gradient (Fig. 4 b, also seen in Fig. 3 a,e,g). The *P. tarandana* specimen in Fig. 3 "d" does not exhibit a strong dark brown row of scales along the termen, however, the scales do exhibit a distinct gradient at the base of the apical teeth of each scale. They appear superficially like

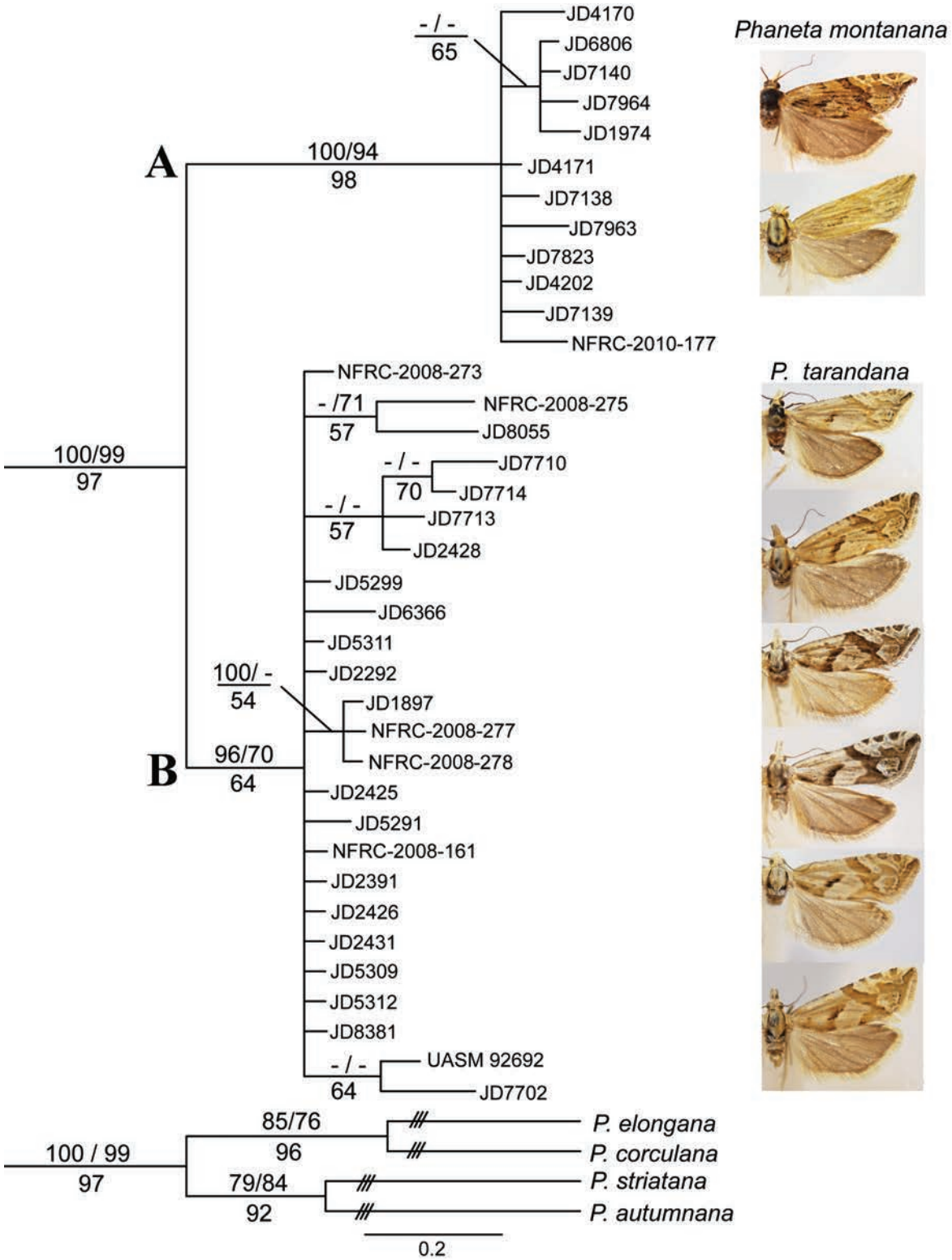

FIG 6. 50% majority rule consensus of 2402 trees generated via Bayesian analysis (120,000 generations) of 631 bp COI for 41 *Phaneta* samples. Numerical values represent parsimony bootstrap support (1000 replicates), and likelihood bootstrap support (100 replicates) above and Bayesian posterior probabilities below. "-" = <50% support. TreeLength=160, CI=0.77, RI= 0.88, HI=0.23.

P. montanana until examined under a microscope since the color of the apical teeth and the stem of the scale are not heavily contrasted as in other *P. tarandana* (Fig. 3 b,f)

Of the 130 specimens examined, 37 fit the description of *P. montanana*, 51 of *P. tarandana*, and 34 appeared to be intermediates, exhibiting diagnostic characters mentioned in both descriptions. An additional eight specimens that were intermediate between *P. montanana* and *P. tarandana* were exceptionally darker brown (dark ground and fascia color) and exhibited some wing streaks.

Variation in genitalic characters was so continuous that characters could not be sorted reliably into distinct character states. Consequently we did not relate genitalic variation to wing pattern variation. In Fig. 5, we show specimens from both *P. tarandana* and *P. montanana* to demonstrate the similarity among genitalia of these species.

DNA sequences. Parsimony and Bayesian trees for 631 nucleotide sites in COI showed two distinct lineages (Fig. 6). COI clade A contained moths that generally resembled the description of *P. montanana*, while those in COI clade B were closer to the description of *P. tarandana*. Additionally, of the 631 sites, 31 sites were parsimony informative. The optimum likelihood tree was partially congruent with this arrangement; COI clade A was monophyletic but placed within a paraphyletic COI clade B. Bootstrapping of the likelihood analysis (100 replicates) supported the monophyly of two distinct clades. Average uncorrected COI sequence divergence between the two major COI lineages (Fig. 6) was 3.12% (min= 2.37% max= 4.18%). Average divergence was 0.56% among the 25 moths in clade B, and 0.36% among the 12 moths in clade A.

Examination of the 521 nucleotide positions in ITS2 sequences revealed only one phylogenetically informative character, a 1 bp indel at ITS2 position 203. This nucleotide position was a cytosine in all 12 specimens characterized as having COI clade A and was coded as a gap in all 25 specimens with COI clade B. Chi-squared analysis showed a statistically significant correlation between COI and ITS2 (X-squared = 23.8138, df = 1, p-value = <0.001). Because the distinction between mitochondrial COI clades A and B is congruent with variation in nuclear ITS2 sequences, we hereafter refer to DNA clades A and B without restricting the names to particular loci.

Integration of morphology and DNA variation. All but one (NFRC-2010-000177, Ft. Assiniboine, AB) of the moths that were resolved in clade A could be identified as *P. montanana* based on wing pattern; all were yellowish-tan and possessed wing streaks, NFRC-2010-000177 was exceptionally darker brown. Moths resolved in clade B ranged in appearance from typical *P. tarandana*, a light form of *P. tarandana*, as well as moths that are intermediate between the two species descriptions. In particular, pale wing coloration, considered to be diagnostic of *P. montanana*, was also represented in clade B in seven specimens. Several specimens of *P. tarandana* exhibited weak wing streaking (See Table 1.0). Values in table 1.0 are based on moths that we had both ITS2 and COI data for (10 *P. montanana*, 18 *P. tarandana*); Moths that had sequence data for only one locus were not included.

The four wing characters that were scored in all specimens showed varying degrees of conformance to the DNA clades. Fascial color character states showed no significant relationship to the two DNA clades (χ^2= 1.61, df = 1, p-value = 0.20). Ground color did correlate significantly with DNA clades (χ^2= 11.10, df = 2, p-value = <0.001) (See Table 1.0)

Wing streaks occurred in members of both DNA clades (Fig. 6). Although present in all members of clade A, wing streaks were also present to some degree in 6 of the 25 members of clade B. Chi-squared analysis showed a significant association between moths with wing streaks and those with clade A DNA (χ^2= 22.16, df = 2, p-value = <0.001).

There was also a significant association between wing fringe scale detail and DNA clades (χ^2= 18.56, df = 3, p-value = <0.001). However, members of DNA clade A predominantly expressed this character as state 3, which lacks a distinct gradient in individual terminal scales. Members of clade B expressed this character as either a distinct band (state 1) or lighter forms that lacked the median band completely (state 2).

In summary, although uncommon, some specimens of *P. tarandana* have lighter wing forms, and in these cases their wing features are less distinct and the fringe is light with no visible dark gradient on individual scales of the termen. *Phaneta montanana* differs from *P. tarandana* in that it lacks the distinct gradient on individual scales on the wing fringe except in one exceptionally darker moth. *Phaneta montanana* wings are also always streaked, a character that is also present in some specimens of *P. tarandana*.

Since the preponderance of morphological evidence supported the existence of two evolutionarily distinct lineages, as initially defined by clades A and B in the molecular analyses, from this point forward we refer to these lineages as *P. montanana* (clade A) and *P. tarandana* (clade B).

Diagnostic characters. The relationship between morphological characters and COI+ITS2 lineages was used to evaluate which character states were most

TABLE 1. Utility of characters in delimiting *P. tarandana* and *P. montanana*. Morphological characters were only assessed in specimens where COI and ITS2 data was available (10 *P. montanana* and 18 *P. tarandana*)

Character	Description of character states		Utility
	P. montanana	*P. tarandana*	
Wing ground color	**9/10** yellowish to light tan **1/10** brown	**11/18** white **5/18** yellowish to light tan **2/18** brown.	Sometimes
Wing fascia color	**6/10** yellowish to tan **4/10** dark brown to grayish	**5/18** yellowish to tan **13/18** dark brown to grayish	No
Forewing fringe scale detail	**8/10** lack distinct band before apical teeth on fringe scales; rather the dark band blends into the apical teeth. **1/10** distinct dark band present just below apical teeth in one exceptionally dark specimen **1/10** too worn to assess	**10/18** possess distinct dark band on the forewing fringe scales, not extending into the apical teeth. **7/18** lack dark pigments on fringe scales, but distinct band on scale still present with less contrast between apical teeth and band **1/18** too worn to assess	Yes
Forewing Streaking	**10/10** wings streaked	**1/18** with streaked wings **6/18** wing slightly streaked, but noticeably less than *P. montanana* **9/18** wing streaks absent **2/18** could not assess, wings greased.	Sometimes
Male Genitalia	*P. montanana* and *P. tarandana* indistinguishable. Angle of sacculus variable, simple rounded "C" shaped cucullus, no modified spines present, phallus simple, short fingerlike socii. Cornuti present in virgin males.		No
Habitat and location	*P. tarandana* and *P. montanana* found across Alberta in sagebrush habitats.		No
Flight time	May 19– Sept. 2	May 31–Aug. 20	No

useful to identify specimens in the absence of molecular information. Apical wing scale coloration, wing streaks, and fascia color were all significantly correlated with the two DNA lineages in the subset of 37 specimens for which DNA was sequenced. When only morphological characters were considered in all available specimens, there were 84 putative *P. tarandana*, and 38 putative *P. montanana*.

Excluding the light forms that do not possess dark scales along the termen, all *P. tarandana* exhibited terminal scales that had a definite origin of the dark gradient at the base of the apical teeth (Fig. 2). All but one *P. montanana* (NFRC-2010-000177) possessed dark scales along the termen that lacked a definite origin of the dark gradient as seen in *P. tarandana*. Evaluation of the angle of the tornus and apex showed no significant difference between the two species.

All specimens were male, excluding two individuals of *P. montanana*. Genitalia showed extensive overlap in variation between *P. tarandana* and *P. montanana*, including in valve shape and length, cucullus shape, and the angle of the sacculus (Fig. 5). Therefore no consistent genitalic characters distinguished putative *P. montanana* from putative *P. tarandana*. In addition, genitalia of either species were indistinguishable from those of *P. transversa* (sensu Heinrich 1923).

For the following two sections, species were determined as above, using fascia color, apical wing scale detail, streaking on the forewings and, when available, COI+ITS2 DNA sequences.

Flight time and habitat association. Flight times overlap extensively between *P. tarandana* and *P. montanana* (Table 1). Both species have two flight peaks each year, one in late spring and one later in summer.

Gilligan et al. (2008) also suggests that *P. montanana* may have 2 broods per year.

Phaneta montanana tends to occur in lowland dune habitat; however, the species has been sampled from higher elevation sagebrush habitats together with *P. tarandana*. There are fewer *P. montanana* specimens from the Rocky Mountain suture zone (Remington 1968) but the two species cannot be reliably delimited based on locality (Fig. 1). In two cases, both species were sampled from the same location on the same night. This occurred in Tranquille Ecological Reserve, British Columbia in September and again in Kootenay Plains Ecological Reserve, Alberta, in August. The seven light form specimens of *P. tarandana* completely lacked dark scales along the termen (unlike any *P. montanana*). This character combination was also seen in four *P. tarandana* from Cypress Hills, one of which appeared to be completely amelanistic, as well as one specimen from Pakowki dunes and two from Waterton Lakes National Park (Crandell campground and the Wishbone trailhead).

Some of our specimens fit Wasingham's (1895) description and illustration of *P. transversa* in that they express markings much more distinctly than typical *P. tarandana*. However, these specimens lack the complete dark subbasal patch illustrated by Walsingham (1895) for *P. transversa*. Neither forewing fringe coloration nor DNA support these specimens as representing a lineage distinct from *P. tarandana*.

Discussion

The *P. tarandana* complex remains a challenging group of moths due to poorly defined species boundaries. Both species treated here presumably feed on sagebrush (Brown 2005) and overlap in distributions. Genitalia, flight time and geographic location have low utility in separating these species. Nonetheless, COI and ITS2 sequences demonstrate that *P. tarandana* and *P. montanana* are distinct but closely related. Most specimens can be diagnosed using wing fringe coloration, as corroborated using molecular markers. Forewing fringe scales on most *P. tarandana* have a distinct dark band below the apical teeth that suffuses into the root of the scale, but light forms of this species have a wing fringe band that is less contrasted with the apical teeth. *Phaneta montanana* generally do not possess a distinct band on the fringe scales.

Walsingham (1895) did not have enough material to draw conclusions about the range of *P. transversa* but he implied that *P. transversa* was a southern form of *P. tarandana*. This could be resolved by examining specimens from nearer to the type locality of *P. transversa* in Colorado.

Our data supports the current taxonomy of *P. tarandana* and *P. montanana* and justifies the continued treatment of both as separate species. The geographical and temporal overlap of *P. tarandana* and *P. montanana* may still allow some gene flow between them, explaining the presence of intermediate moths. However, the fact that most specimens are clearly one species or the other suggests that members of both lineages are able to maintain their genomic integrity, satisfying both the genomic integrity and general lineage species concepts (Sperling 2003; De Quieroz 1998).

Acknowledgements

We thank Greg Pohl (Canadian Forest Service, Northern Forestry Centre) and Charley Bird (private collection) for generous loans of specimens, Richard Brown and Don Wright for inspiring conversation, and Danny Shipeley for assisting with photography of genitalia slides. We are also grateful to members of the Sperling lab for intellectual support, including Sarah Leo, Bryan Brunet, Heather Bird, Marla Schwarzfeld, Julian Dupuis, and Jasmine Janes. Cameron Lockerbie, Wayne Nordstrom, and Cyndi Smith facilitated permits in protected areas and provincial and federal parks. Finally we acknowledge funding from an Alberta Conservation Association grant to JD & FS and an NSERC Discovery Grant to F. Sperling.

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Submitted for publication 28 November 2012; revised and accepted 4 March 2013