The Reliability of Genitalia Morphology to Monitor the Spread of the Invasive Winter Moth (Lepidoptera: Geometridae) in Eastern North America

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Abstract
Winter moth, Operophtera brumata L. (Lepidoptera: Geometridae), causes widespread defoliation in both its native and introduced distributions. Invasive populations of winter moth are currently established in the United States and Canada, and pheromone-baited traps have been widely used to track its spread. Unfortunately, a native species, the Bruce spanworm, O. bruceata (Hulst), and O. bruceata × brumata hybrids respond to the same pheromone, complicating efforts to detect novel winter moth populations. Previously, differences in measurements of a part of the male genitalia called the uncus have been utilized to differentiate the species; however, the accuracy of these measurements has not been quantified using independent data. To establish morphological cutoffs and estimate the accuracy of uncus-based identifications, we compared morphological measurements and molecular identifications based on microsatellite genotyping. We find that there are significant differences in some uncus measurements, and that in general, uncus measurements have low type I error rates (i.e., the probability of having false positives for the presence of winter moth). However, uncus measurements had high type II error rates (i.e., the probability of having false negatives for the presence of winter moth). Our results show that uncus measurements can be useful for performing preliminary identifications to monitor the spread of winter moth, though for accurate monitoring, molecular methods are still required. As such, efforts to study the spread of winter moth into interior portions of North America should utilize a combination of pheromone trapping and uncus measurements, while maintaining vouchers for molecular identification.

Key words: forest pest, biological control, microsatellite, morphology

As human-mediated transport of materials has increased over the past century, the frequency of introductions of invasive species has also increased (Meyerson and Mooney 2007, Roques et al. 2009). These continuous introductions, coupled with the increased severity of species invasions as a result of climate change, have had widespread negative effects on ecosystems across the globe (Dukes 2000). For invasive insects, a breakthrough in the monitoring of their spread came about with the isolation of insect sex-pheromones and their synthesis into commercially available pheromone-baited traps (Brockerhoff et al. 2006, El-Sayed et al. 2006, Bogich et al. 2008). Pheromone traps have an ability to detect the presence of insects at densities far lower than that achieved by any other sampling method, making them particularly powerful for detecting novel populations of invasive species. In addition, sex-pheromones can be highly species-specific with different species in the same genus, often employing different minor components or different ratios of components to avoid hybridization (Adams and Tsutsui 2020).

While the prevention of the establishment of invasive species is always the principal management option, once a non-native species has invaded a novel habitat, effective monitoring methods are required to implement efficient control measures (Crowl et al. 2008). Biological control is one method that has been used to successfully mediate habitat destruction, prevent irreversible reductions in population sizes of native species, and reduce economic costs from invasive species introductions (van Driesche 2012). Under this approach, predators, parasites, or pathogens are introduced or promoted to reduce the abundance of the invasive species (DeBach and Rosen 1991). However, for biological control methods to be effective and safe, long-term monitoring programs with efficient methods for
documenting the presence of an invasive species are required (Mills 2000).

Winter moth, *Operophtera brumata* L. (Lepidoptera: Geometridae), is an invasive species to North America that causes widespread defoliation to deciduous trees and fruit crops (Elkinton et al. 2015). Winter moth first became established in North America in the 1930s in Nova Scotia, Canada (Embree 1966). Since then, it has been reported in Oregon in the 1950s (Kimberling et al. 1986), Washington and British Columbia in the 1970s (Roland and Embree 1995), and more recently in the northeastern United States in the 1990s (Elkinton et al. 2015). While successful biological control programs have been initiated in each region of establishment (Elkinton et al. 2018), the population in the northeast appears to be spreading rapidly from the coasts to the interior portions of this region (Elkinton et al. 2014), suggesting that this invasive population might have the potential to cause much more widespread habitat destruction than previously established populations have done. Unfortunately, documenting the spread of winter moth, quantifying its environmental impacts, and enacting efficient management strategies is complicated by the fact that adults closely resemble and actively hybridize with a native relative, the Bruce spanworm, *O. bruceata* (Hulst) (Elkinton et al. 2010, 2011, 2015; Havill et al. 2017).

Previous efforts to monitor the spread of winter moth have used pheromone-baited traps to capture winter moth along an east-west transect in central Massachusetts (Elkinton et al. 2010, 2011, 2015) and from locations across North America (Andersen et al. 2019). The winter moth sex-pheromone was first identified by Roelofs et al. (1982) and is unusual compared to most Lepidoptera in that it consists of a single compound (Zhang 2016). Multi-compound pheromones are thought to improve the specificity of the attraction signal, so a likely result of having a simplified sex-pheromone is that we have readily captured Bruce spanworm in traps baited with the winter moth sex-pheromone (Elkinton et al. 2010, 2014). Consequently, all subsequent studies of winter moth prevalence conducted by our laboratory have utilized traps baited only with the winter moth pheromone.

Due to the inevitable collection of Bruce spanworm males, our efforts to document the spread of winter moth and its hybridization with Bruce spanworm have relied on molecular methods including fragment length polymorphisms, DNA sequencing, or genotyping with microsatellites (e.g., Elkinton et al. 2010, Havill et al. 2017, Andersen et al. 2019). While highly accurate, these methods are not always practical, particularly in comparison to rapid field-based identifications. Previously, morphological analyses have been used to distinguish these two species. For example, adult females have been suggested to be identifiable to species based on differences in wing bud sizes (Troubridge and Fitzpatrick 1993), and for males, differences in wing sizes and genitalia (Fig. 1) (Troubridge and Fitzpatrick 1993, Elkinton et al. 2010) have been proposed, though the accuracy of either method has not been verified, and an earlier study cautioned that there were few reliable differences between these two species (Eidt et al. 1966).

Given the relative ease of collecting adult winter moth, Bruce spanworm, and hybrid males through the use of pheromone-baited traps at dusk, we were interested in testing the accuracy of morphological measurements of the male genitalia in providing species-level identifications. We then compared the results from the morphological analyses to the molecular analyses to estimate the accuracy of these morphological measurements for species-level identifications.

![Fig. 1. Photographs of uncus from the genitalia of (A) winter moth (*Operophtera brumata*) and (B) Bruce spanworm (*O. bruceata*). Horizontal dashed lines indicate where mid (m) and tip (t) uncus width measurements were taken.](https://academic.oup.com/ee/article/49/6/1492/5933726)
Methods

Sample Collection
Moths were collected using pheromone-baited traps for adult males of both winter moth and Bruce spanworm (Elkinton et al. 2010, 2011) along an established transect along Route 2 in Massachusetts (Fig. 2) that is predominantly Bruce spanworm in the West and winter moth in the East with high rates of hybridization along a stable hybrid zone near the center of the transect (Elkinton et al. 2010, Andersen et al. 2019). In the winter of 2018, moths were collected from traps four times during the flight of the male moths, every 2 wk from 29 November 2018 to 13 January 2019. After collection, moths were counted and placed into coin envelopes and stored at −20°C.

Morphological Analysis
Ten male moths were randomly selected from each trap/week collection. The wings were removed and placed in glassine envelopes to be retained as vouchers, and the posterior section of the abdomen was dissected and the uncus removed and slide-mounted using cellulose-based Insect Repair Adhesive (Bioquip Products). The rest of the body was placed in a 1.5 ml tube and stored at −80°C for later DNA extraction (see below). Following Elkinton et al. (2010), measurements from the uncus (i.e., a sclerotized structure formed from the major portions of tergum 10 with an element of the tegumen that is directed downward, shielding the aedeagus; Smith 1906, Scoble 1992), were taken from the point just before the uncus begins to curve (hereafter the ‘tip’) and from the lateral midpoint (hereafter the ‘middle’; see Fig. 1). Species were delimited using the following criteria: uncus tip measurements >132 μm are classified as winter moth, uncus tip measurements <108 μm are classified as Bruce spanworm. Operationally, uncus tip measurements 108 μm ≤ × ≤ 132 μm are classified as hybrids (G. H. Boettner, Personal Communication). Troubridge and Fitzpatrick (1993) report similar measurements, with winter moth males having uncus width measurements of ca. 140 μm, and Bruce spanworm males having uncus width measurements of ≤120 μm.

DNA Extraction and Microsatellite Amplification
Genomic DNA was extracted from each male moth using the Omega E.Z.N.A tissue DNA kit following the manufacturer’s protocol as previously reported (Havill et al. 2017, Andersen et al. 2019). Twelve directly labeled microsatellite loci were amplified using the multiplex combinations outlined in Havill et al. (2017). All PCR reactions

Fig. 2. The total number of genotyped Bruce spanworm (black), hybrids (gray), and winter moth (white) individuals (top) at each surveyed trap along Route 2 in Massachusetts, and trap locations (bottom) drawn in ArcGIS v.10.3.1 (ESRI, Inc.).
were based on 10 μl reactions containing 2 μl of 5X PCR Buffer (Promega), 0.8 μl of 25 mM MgCl2 (Promega), 1 μl of dNTP mix (10 mM each; New England BioLabs), 0.25 μl of fluorescently labeled forward primers (10 nM of each forward primer in a group), 0.25 μl of reverse primers (10 nM of each reverse primer in a group), 0.10 μl GoTaq G2 Flexi DNA polymerase (Promega), and 1.0 μl sample DNA template. Thermocycler conditions were as follows; after an initial denaturation step, a touchdown protocol was used with a starting annealing temperature of 61°C, that was decreased 2°C each cycle for 5 cycles, then held constant at 51°C for 30 cycles (Havill et al. 2017). The PCR products were sent to the DNA Analysis Facility on Science Hill at Yale University for genotyping in cycles (Havill et al. 2017). The PCR products were run on a 3730xl DNA Analyzer (Thermo Fisher Scientific). Fragment lengths were scored using the microsatellite plugin in the software program Geneious v. R11 (BioMatters, Fisher Scientific). Comparisons to the GeneScan 500 LIZ size standard (Thermo Fisher Scientific Facility on Science Hill at Yale University for genotyping in cycles (Havill et al. 2017). The PCR products were sent to the DNA Analysis Facility on Science Hill at Yale University for genotyping in cycles (Havill et al. 2017). The PCR products were run on a 3730xl DNA Analyzer (Thermo Fisher Scientific). Fragment lengths were scored using the microsatellite plugin in the software program Geneious v. R11 (BioMatters, Fisher Scientific). Comparisons to the GeneScan 500 LIZ size standard (Thermo Fisher Scientific).

Molecular Identifications

The probability of assignment (Q) of sampled individuals to one of two distinct genetic clusters (K) was calculated using STRUCTURE v.2.3.2 (Pritchard et al. 2000, Falush et al. 2003) based on 10 independent analyses run using the admixture model, correlated allele frequencies, and default settings, with random starting values, runtimes of 1,000,000 generations, and burn-in periods of 100,000 generations. Results were then summarized across runs using CLUMPAK (Kopelman et al. 2015). Individuals with scores of Q ≥ 0.75 or Q ≤ 0.25 were classified as either Bruce spanworm or winter moth, respectively, and individuals with scores of 0.25 < Q < 0.75 were classified as hybrids following Andersen et al. (2019).

Accuracy of Morphological Methods

To determine the accuracy of the uncus thresholds presented in Troubridge and Fitzpatrick (1993), we first averaged the middle and tip measurements as the authors do not specify what part of the uncus should be measured. We then used the average measurement to assign species using the thresholds described above (Note: Troubridge and Fitzpatrick do not provide measurement recommendations for hybrids; therefore, we assume that intermediate values are hybrids, though we acknowledge that the authors may not have intended their thresholds to be used as such). To determine the accuracy of the uncus measurements, we assign species (as described above), and then calculated Type I and Type II error rates. To calculate Type I error rates (i.e., the probability of having false positives for the presence of winter moth), for each species, we divided the number of correctly identified individuals (i.e., those individuals whose morphological ID to a particular species were in agreement with their molecular ID), by the total number of individuals assigned to that species based on uncus measurements, and subtracted that value from 1. To calculate Type II error rates (i.e., the probability of having false negatives for the presence of winter moth), for each species, we divided the number of correctly identified individuals (as above), by the total number of individuals assigned to that species based on molecular genotyping, and subtracted that value from 1.

Statistical Assessment

Using the molecular assignment results, differences in measurements of the tip of the uncus, the middle of the uncus, as well as differences in the averages of these two measurements, and differences in the ratio of tip measurement divided by the middle measurement were determined using an analysis of variance (ANOVA) test as implemented in R v. 3.6.2 (R Core Team 2019). Post hoc pairwise-comparisons were estimated using Tukey's Honest Significant Difference test, as implemented in R, and because multiple tests were performed (i.e., tip, middle, average, ratio), we used Bonferroni's correction to estimate an adjusted alpha of 0.0125 (α = 0.05/n = 4 comparisons) when presenting significance.

Results

Sampling

We obtained morphological measurements and molecular identifications for 808 moths collected along Route 2 in Massachusetts during our four collection events. This included 201 moths from the first collection event, 249 moths from the second collection event, 225 moths from the third collection event, and 133 moths from the final collection event. The number of moths of each species at each trap is shown graphically, along with the locations of each collection event in Fig. 2.

Molecular and Morphological Identifications

Molecular identifications based on summary of the 10 independent structure runs indicated that 528 samples were identified as Bruce spanworm, 253 were identified as winter moth, and 27 were identified as hybrids (assignment scores for each individual are provided in Supp Appendix S1 [online only]). Based on the cutoffs presented in Troubridge and Fitzpatrick (1993), 534 samples were identified as Bruce spanworms, 136 were identified as hybrids, and 128 were identified as winter moth. The Type I error rate based on the authors method was 8.8% for Bruce spanworm, 1.6% for winter moth, and 98.5% for hybrids, and the Type II error rate was 6.9% for Bruce spanworm, 49.4% for winter moth, and 92.3% for hybrids (Table 1). Based on the cutoffs used in Elkinton et al. (2010), 428 samples were identified as Bruce spanworms, 208 were identified as hybrids, and 162 were identified as winter moth. The Type I error rate based on the authors method was 5.6% for Bruce spanworm, 5.6% for winter moth, and 95.2% for hybrids, and the Type II error rate was 22.8% for Bruce spanworm, 38.6% for winter moth, and 61.5% for hybrids (Table 2).

| Table 1. Type I and Type II error rates for assignments based on Troubridge and Fitzpatrick (1993) compared to microsatellite genotype results |
|--------------------|----------------|----------------|----------------|
| Molecular Uncus | Troubridge and Fitzpatrick |
| | Bruce spanworm | Hybrids | Winter moth | Type II error |
| Bruce spanworm | 487 | 35 | 1 | 6.9% |
| Hybrids | 23 | 2 | 1 | 92.3% |
| Winter moth | 24 | 99 | 126 | 49.4% |
| Type I error | 8.8% | 98.5% | 1.6% |

Type I error rates were calculated for each species by dividing the number of correctly identified specimens by the total number of specimens assigned to that species based on uncus measurements, and subtracting that value from 1 (e.g., for Bruce spanworm; 1 – [487/534] = 8.8%). Type II error rates were calculated for each species by dividing the number of correctly identified specimens by the total number of specimens assigned to that species based on molecular measurements, and subtracting that value from 1 (e.g., for Bruce spanworm; 1 – [487/523] = 6.9%).
For Bruce spanworm, the average measurements (±SE) for the middle of the uncus was 100.2 μm ± 0.8 μm, the tip of the uncus was 95.4 μm ± 0.9 μm, the average of the two measurements was 98.7 μm ± 0.6 μm, and the ratio of the two measurements was 0.97 ± 0.01. For winter moth, the average measurements (±SE) for the middle of the uncus was 139.7 μm ± 1.9 μm, the tip of the uncus was 134.7 μm ± 1.8 μm, the average of the two measurements was 139.4 μm ± 1.2 μm, and the ratio of the two measurements was 0.96 ± 0.01. For hybrids, the average measurements (±SE) for the middle of the uncus was 100.6 μm ± 4.8 μm, the tip of the uncus was 105.3 μm ± 5.2 μm, the average of the two measurements was 106.9 μm ± 2.6 μm, and the ratio of the two measurements was 1.06 ± 0.03.

Table 2. Type I and Type II error rates for assignments based on Elkinton et al. (2010) compared to microsatellite genotype results

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<th>Molecular \ Uncus \ Type II error</th>
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Type I and Type II error rates were calculated as per Table 1.

Statistical Assessment

Highly significant differences were observed between species based on measurements of the middle ($F = 537.2, df = 2, P < 2e-16$), and the tip of the uncus ($F = 594, df = 2, P < 2e-16$), as well as the average of the two measurements ($F = 328.8, df = 2, P < 2e-16$). However, there were no significant differences between species based on the ratio of the two measurements after applying Bonferroni’s correction ($F = 3.633, df = 2, P = 0.0269$) (Fig. 3). Pairwise comparisons indicated that winter moth was always significantly differentiated from Bruce spanworm and hybrids, but only by using the measurement based on the tip of the uncus could all three classifications be differentiated (Fig. 3).

Discussion

Detecting novel populations of invasive species and coordinating local and regional programs to control these populations is one of the most important tools for reducing the spread of invasive species (Fitzpatrick et al. 2009). To aid these efforts, programs have integrated methods such as species distribution modeling (Gormley et al. 2011) and citizen-scientist observations (Dickinson et al. 2010, Goczal et al. 2017) to detect novel populations. However, increasingly researchers are examining the effects of data precision and accuracy of classifications on the ability to enact effective invasive species control programs (Cheney et al. 2018, Bonneau et al. 2019). Accuracy becomes increasingly important when native congeners of an invasive species are present, as misidentifications could inadvertently result in wasteful expenditure of resources (when the native species is misidentified as the invasive) or the failure to establish control programs (when the invasive species is misidentified as the native). Here we find...
that morphological-based measurements aimed at differentiating the invasive winter moth and the native Bruce spanworm differed significantly between these two species, and if measurements of the tip of the uncus were used, between hybrids as well (Fig. 3).

Using these measurements, classifications separating Bruce spanworm and winter moth have extremely low Type I error rates (i.e., the probability of having false positives for the presence of winter moth); however, they have much higher Type II error rates (i.e., the probability of having false negatives for the presence of winter moth), and were completely incapable of identifying hybrid individuals (Tables 1 and 2). As such, morphological-based identifications could play a role during preliminary surveys aimed at monitoring the spread of winter moth, particularly into coastal regions of the Mid-Atlantic states and Appalachia where recent species distribution modeling suggests winter moth could become established (Blackburn et al. 2020). Based on our results, when conducting morphological identifications of winter moth, Bruce spanworm, and their hybrids, we recommend using measurements from the tip of the uncus, as these measurements were most clearly distinct between species (Fig. 3b). Our results suggest that tip measurements of ~95 μm likely signify Bruce spanworm, and that tip measurements of ~135 μm likely signify winter moth. However, given that uncus measurements were unable to detect hybrid individuals and that hybrids may play an important role in both overcoming Allee effects (Ward et al. 2008) and in range expansion (Coulter et al. 2020, Manzoor et al. 2020), these measurements should be coupled with molecular identifications when possible, to reduce Type II errors. Detecting hybrid individuals may be particularly important for studying the spread of winter moth as we previously detected a hybrid individual in the Great Lakes regions using microsatellite genotyping techniques (Andersen et al. 2019).

Conclusion

Here, we find that uncus measurement ranges can be used to accurately identify pure Bruce spanworm and pure winter moth individuals, but not their hybrids. We recommend that future winter moth monitoring programs use uncus tip measurements to differentiate these two species, as this measurement showed significant differences between both species and hybrids, and that voucher specimens be retained for subsequent molecular identifications as needed.

Supplementary Data

Supplementary data are available at Environmental Entomology online.

Supplemental Appendix S1: Structure formatted genotype scores for each individual, locality information, and species-level identifications.

Acknowledgments

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