# Considerations for Accurate Identification of Adult *Culex restuans* (Diptera: Culicidae) in Field Studies

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**ABSTRACT** Understanding the ecology and behavior of different mosquito species (Diptera: Culicidae) is essential for identifying their role in disease transmission cycles and public health risk. Two species of *Culex* mosquitoes in the northeastern United States, *Culex pipiens* L. and *Culex restuans* Theobald, have been implicated in enzootic transmission of West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV). Despite the difficulty of differentiating these two species as adults, many public health workers and vector biologists collecting adults in the field separate these species based on external morphology. This approach is often used rather than examination of dissected male genitalia or polymerase chain reaction (PCR)-based diagnostics due to time or cost constraints. We evaluated the reliability of seven published morphological characters to differentiate adults of these species by comparing blindly scored morphology with PCR-based confirmations. Our study demonstrates that morphological identification of *Cx. pipiens* is marginal and often not reliable for *Cx. restuans*. We also examined error rates with molecular-based approaches. DNA samples were contaminated with as little as one leg from another species. We conclude that to fully understand the respective roles of *Culex* species in the epidemiology of WNV and other pathogens, more attention should be paid to these considerations for accurate species identification.

**KEY WORDS** morphology, West Nile virus, *Culex pipiens*, *Culex restuans*, surveillance

*Culex* mosquitoes (Diptera: Culicidae) are among the most important vectors of West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) in North America (Andreadis et al. 2001, Turell et al. 2001). In New York state, *Culex pipiens* L. and *Culex restuans* Theobald often inhabit similar breeding sites, and they are readily collected in Centers for Disease Control and Prevention (CDC) miniature light traps and gravid traps for WNV surveillance programs. *Cx. restuans* has a broad range in North America from Canada into Mexico (Carpenter and LaCasse 1955), and with reports as far south as Guatamala and Honduras (Strickman and Darsie 1988). Although host-feeding patterns of *Cx. pipiens* have been well studied, relatively little is known about *Cx. restuans* (Tempelis 1975, Magnarelli 1977). *Cx. restuans* are assumed to be ornithophilic and similar to *Cx. pipiens.* It has become increasingly apparent that *Cx. restuans* may play an important role in the transmission dynamics of West Nile virus in eastern North America (Andreadis et al. 2004, Ebel et al. 2005, Kilpatrick et al. 2005, Darbro and Harrington 2006).

Unfortunately, these two species are often difficult to differentiate as adults, and they are frequently lumped together in WNV surveillance pools or they may be inadvertently misidentified by even the most experienced mosquito biologists. A percentage of *Cx. restuans* emerge with two white spots on the scutum. These distinguishing scales are easily lost during jostling in mosquito traps or handling before identification. Furthermore, most *Cx. restuans* emerge without these distinguishing spots (Fig. 1) (Michener 1947, Wood et al. 1979, Means 1987). Although the difficulty in identification of these two species as adults has been recognized for decades (Michener 1947), the major taxonomic keys (Darsie and Ward 1981, 2005; Means 1987; Andreadis et al. 2005) used by mosquito researchers ignore this issue and provide users with external morphological traits for identification.

Apperson et al. (2002) and others (Andreadis et al. 2005) reported accurate *Cx. pipiens* and *Cx. restuans* identification by using a group of additional external morphological characters. They based their conclusion on PCR confirmation with a small subset of samples ($n = 45$).

One reliable morphological trait is examination of male genitalia (Carpenter and LaCasse 1955). Unfortunately, this requires dissection of each male sample, and it does not allow accurate identification of females, the predominant sex captured in carbon dioxide-baited or gravid traps, and the main focus of vector-borne disease studies. Thus, many programs rely on the morphological traits described by Apperson et

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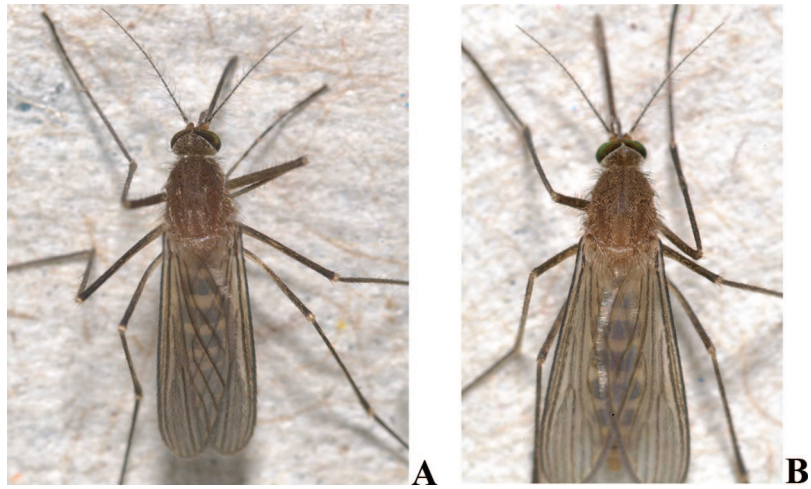


**Fig. 1.** The presence of white dots on scutum of *Cx. restuans* (A) is a reliable character for this species when present. (B) No spots are found on the scutum of *Cx. pipiens.*

al. (2002) and others (Darsie and Ward 1981, 2005; Means 1987; Andreadis et al. 2004, 2005).

Based on our own observations in the field, we began to question the reliability of these morphological characters. Our goal was to robustly test the utility of morphological traits to identify the most reliable approaches for our field studies of *Cx. pipiens* and *Cx. restuans.*

## Materials and Methods

**Mosquitoes.** Adults were obtained from a variety of field collection methods for blind evaluation of adult morphological characters. Larvae were collected from the field to obtain adults of known species (determined from larval traits).

**Collection of Adults.** *Culex* mosquitoes were obtained from sparrow- and chicken-baited traps as described by Darbro and Harrington (2006), from dairy farm hedgerow (42°26.501′ N 076°25.889′W) and from vegetation aspirator collections at public parks (42° 28.532′ N 076° 27.044′ W), golf courses (42°27.486′ N 076° 27.594′ W) and private residences (42° 26.924′ N 076° 25.757′ W) in central New York (L.C.H. et al. unpublished data) in 2002.

**Collection of Larvae.** Egg rafts were collected from a used tire pile (42° 31′52″ N, 76° 19′41″ W) on 1 August 2005, and they were carefully examined under a dissecting scope to determine viability. Unhatched rafts were placed into 120-ml cups with 3 ml of diet (1:2:1 fish food/rabbit pellets/bovine liver powder) and 80 ml of deionized water. Each raft was held separately at 20°C, and after hatch, second–fourth instars were identified using standard keys (Darsie and Ward 1981, 2005, Means 1987) to confirm as *Cx pipiens* or *Cx. restuans.* Larvae of each species were reared to adults in trays of 200 larvae per tray. Four days after emergence, all females were removed, frozen at −10°C, placed in 1.5-ml sterile tubes, and given a unique code known only to R.L.P. In addition, specimens from a New York state colony of *Cx. pipiens* females established in 2003 and augmented biannually with new field material were aspirated out of cages, frozen at −10°C, and placed individually in labeled 1.5-ml sterile tubes. When female *Cx. restuans* (identified to species as larvae) emerged from field-collected egg rafts, they were separated and frozen in sterile tubes as described above.

**Morphological Identification of Field-Collected Adults.** Adult *Culex* mosquitoes from bird-baited traps and resting collections were placed in sterile 1.5-ml tubes or in petri dishes (Fisher, Agawam, MA) on filter paper, wrapped in plastic film, and stored at −20°C until further processing. *Cx. pipiens* and *Cx. restuans* were identified to species by using morphological traits in Table 1 as described by Apperson et al. (2002). We did not include specimens that had scutal spots, because this is a reliable character if present. All specimens were examined under a dissecting microscope, and they were illuminated with a fiber optic light source at uniform light intensity. After identification, individual mosquitoes were placed in sterile microcentrifuge tubes for DNA extraction described below.

**Morphological Identification of Mosquitoes Collected as Larvae.** Mosquitoes that were positively identified as *Cx. restuans* or *Cx. pipiens* as larvae from field collections following the standard keys (Darsie and Ward 1981, Means 1987, Andreadis et al. 2005) served as a morphological control for our experiment. After identification, larvae were reared to adults by R.L.P. and randomly placed in 1.5-ml tubes with no indication of their correct species. If any *Cx. restuans* emerged with white scutal spots, the spots were rubbed off by RLP with a fine paintbrush. All mosquito samples were provided blindly to L.C.H. for identification to species. Voucher specimens for each species were deposited in Cornell University's Insect Collection (http://www.entomology.cornell.edu/CUIC/).

**PCR-Based Confirmation of Identifications.** Identifications of all *Cx. pipiens* and *Cx. restuans* were

**Table 1. External morphological traits used to identify *Cx. pipiens* and *Cx. restuans* and analysis of agreement with PCR-confirmed identification (Kappa statistics and 95% CI)**

| Character | Source | *Cx. restuans* | *Cx. pipiens* | Kappa statistic | 95% CI |
|---|---|---|---|---|---|
| 1. Integument color | Apperson et al. (2002) | Reddish brown | Tan to brown | 0.169 | 0.055–0.278 |
| 2. Scutal scale color | Carpenter and LaCasse (1955), Apperson et al. (2002) | Dark brown | Light brown | —[a] | —[a] |
| 3. Scutal scale shape | Apperson et al. (2002) | Not curved or falcate | Curved or falcate | −0.109 | −0.040 to 0.182 |
| 4. Scutal arrangement | Apperson et al. (2002) | Well arranged | Coarse and "scruffy" | −0.010 | −0.040 to 0.020 |
| 5. Scutal scale size | Apperson et al. (2002) | Shorter | Longer | −0.251 | −0.362 to −0.140 |
| 6. Abdominal band color | Apperson et al. (2002) | Usually white | Usually yellow | 0.177 | 0.105–0.249 |
| 7. Abdominal shape and margin | Carpenter and LaCasse (1955); Darsie and Ward (1981, 2005); Means (1987); Andreadis (2004) | Broad, merging with white spot on lateral margin | Convex, constricted near white spot with lateral margin | 0.323 | 0.214–0.433 |

[a] Kappa statistic not calculated for these data, because 47% of samples did not have the character of either species.

determined by PCR following the protocol of Crabtree et al. (1995). DNA was extracted using DNAzol reagent (Invitrogen, Carlsbad, CA) and amplified (Crabtree et al. 1995, 1997). Each 20-$\mu$l PCR tube contained 2.0 $\mu$l of 10× PCR buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl) (Invitrogen), 1.6 $\mu$l of 25 mM $MgCl_2$ (Promega, Madison, WI), 1.0 $\mu$l of 100 mM dATP, 1.0 $\mu$l of 100 mM dCTP, 1.0 $\mu$l of 100 mM dGTP, 1.0 $\mu$l of 100 mM dTTP (all dNTPs were from Invitrogen), 0.4 $\mu$l of 25 nM CP16 universal forward primer (5′-GCGGGTACCATGCTTAAATTTAGGGGGTA-3′), 0.4 $\mu$l of 25 nM PQ10 reverse primer (5′-CCTATGTCCGCGTATACTA), 0.4 $\mu$l of 25 nM R6 reverse primer (5′-CCAAACACCGGTACCCAA-3′), 0.4 $\mu$l of 25 nM S20 reverse primer (5′-TGAGAATACATACCACTGCT-3′) (all primers were from Integrated DNA Technologies, Inc., Coralville, IA), and 0.1 $\mu$l of 5 U/$\mu$l Platinum *Taq* polymerase (Invitrogen). Each sample was heated at 96°C for 4 min, 96°C for 30 s, 55°C for 45 s, and then 72°C for 90 s. This cycle was repeated 39 times, with a final step at 72°C for 4 min. Amplified samples were stored at 4°C.

Amplified DNA was separated on ethidium bromide-stained 1.5% agarose gels for 16 min at 400 V and buffered with 1× sodium boric acid, pH 8.0 (Brody and Kern 2004). Fragment lengths were determined by referencing a 100-bp ladder (Novagen, Madison, WI).

**Cross-Contamination Assay.** To determine limits of accuracy for the PCR-based identification approach, body parts of known *Cx. restuans* and *Cx. pipiens* specimens were homogenized in 38 different combinations to determine qualitatively the strength and potential for double bands of 506 and 698 bp representing both species. Heads, thoraces, abdomens, wings, and legs from mosquitoes of each species were carefully isolated and then allocated in various combinations to sterile tubes. Genomic DNA extraction and amplification were performed as described above. This assay was replicated three times.

**Data Analysis.** Each specimen was given a nominal score of one (*Cx. pipiens* character) or two (*Cx. restuans* character), based on the criteria reported by (Darsie and Ward 1981, 2005; Apperson et al. 2002; Andreadis et al. 2005). If traits were a combination of the two types they were scored as variable, but they were excluded from the data analysis. All morphological data confirmed by PCR were entered in an Excel (Microsoft, Redmond, WA) database. Morphological data were analyzed for character by species agreement by using kappa statistics (Proc Freq, SAS version 9.01; SAS Institute, Cary, NC). This approach is used to quantify the level of agreement compared with chance alone in scored nominal data sets (unordered categorical data) (Landis and Koch 1977).

## Results

**Comparison of Morphological Traits.** Significant variation in morphology was found within 324 examined specimens of *Cx. pipiens* and *Cx. restuans.* Varied traits included integument color (Fig. 2), abdominal band pattern (Fig. 3), and scutal scale pattern and color. In total, 180 *Cx. pipiens* and 121 *Cx. restuans* were blindly examined for the seven morphological traits, including those outlined by (Apperson et al. 2002) (Table 1). Considerable variation in traits was found for *Cx. pipiens* (Fig. 4). All seven characters together accurately predicted the correct species for only 60% of true *Cx. pipiens* examined.

When examined independently, the most reliable traits for *Cx. pipiens* included scutal scale shape (78% accuracy), integument color (76% accuracy), and abdominal band margin (73% accuracy). Although 97% of *Cx. pipiens* had a scutal scale arrangement described as "scruffy" (Apperson et al. 2002), 99% of *Cx. restuans* also looked "scruffy". Consequently, we did not consider this trait useful. Scutal scale size (44% accuracy) and abdominal color (27% accuracy) were the least predictive traits for *Cx. pipiens.*

In contrast, the most reliable traits for *Cx. restuans* were abdominal band color (91% accuracy) and abdominal band margin (57% accuracy) (Fig. 5). However, 73% of *Cx. pipiens* also had the *restuans*-type abdominal band color. The least predictive traits for *Cx. restuans* included scutal scale shape (4% accuracy) and size (<1% accuracy). *Cx. restuans* could be reliably identified with all seven traits only 34% of the time (Fig. 5).

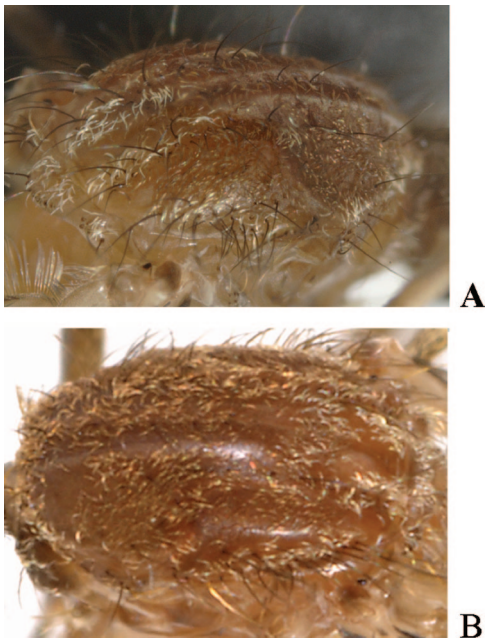


**Fig. 2.** (A) Tan integument (*pipiens* character) (specimen confirmed as *Cx. restuans*). (B) Reddish brown integument (*restuans* character) (specimen confirmed as *Cx. pipiens*).

Considering scoring of both species together in an agreement matrix with classification based on external morphology by true species identity (confirmed by PCR), the greatest agreement was for abdominal shape and margin (K = 0.323, confidence interval [CI] = 0.214–0.433; Table 1.) Kappa values range from −1 to 1 with perfect agreement equal to 1. A Kappa value of 0.60 or higher indicates good to excellent agreement (Landis and Koch 1977).

**Larval Identification Control.** All samples identified as *Cx. restuans* or *Cx. pipiens* in the larval stage using published keys were correct as confirmed by PCR-based confirmation. No differences in blind scoring of morphology were found between adults emerged from field-collected larvae and those captured as adults in traps.

**Cross-Contamination Assay.** Combinations of one, two, and three legs from each species yielded double bands in all mixtures. Nineteen percent of two species combinations yielded amplification of DNA from only one species. This result occurred when fewer than three legs were combined with a head, head and thorax, or whole body of a second species. Double bands, confirming the presence of both species in a mixed sample, were found in cases when three or more *Cx. restuans* legs were combined with a *Cx. pipiens* head and thorax (without legs), or two *Cx. restuans* legs and a *Cx. pipiens* head. Results of a subset of these combinations are presented in Fig. 6.

## Conclusions

Our results demonstrate a lack of accuracy and reliability with external morphological traits used to distinguish *Cx. pipiens* and *Cx. restuans* in New York state. Our results also may extend to other regions of the United States where these two epidemiologically important vectors of West Nile virus overlap. Three of the most commonly used taxonomic mosquito keys (Means 1987, Andreadis et al. 2005, Darsie and Ward 2005), as well as additional morphological traits from published articles (Apperson et al. 2002, Andreadis et al. 2004), distinguish *Cx. pipiens* from *Cx. restuans* based on abdominal tergite scale patterns and other traits, which resulted in a nearly 30% error rate for the specimens we examined.

Apperson et al. (2002) recognized that the color and shape of bands on the abdomen can vary, and it is best to use all seven characters together to identify these species. In our hands, use of these morphological traits allowed us to correctly identify the species only 63%


**Fig. 3.** (A) Constricted abdominal bands (*pipiens* character) on *Cx. pipiens*. (B) Broad, straight abdominal bands on Cx. *restuans*.

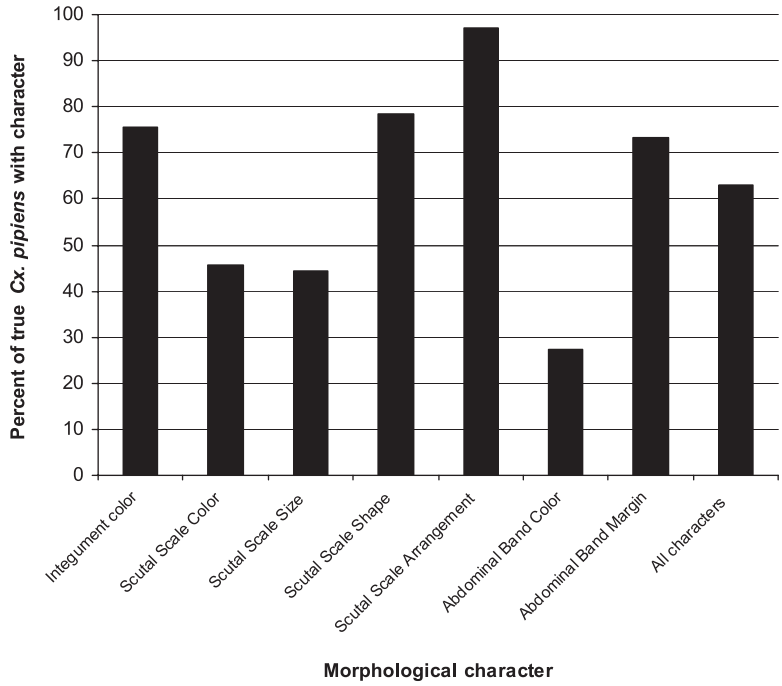


**Fig. 4.** Percentage of PCR-confirmed *Cx. pipiens* that had the correct characters ($n$ = 180). The last column shows percentage of accuracy using all seven morphological characters.

of the time for *Cx. pipiens* and only 34% of the time for *Cx. restuans.* Our error rate for *Cx. restuans* was higher than the 20% error rate determined by Apperson et al. (2002). Among the putative traits we examined, the two most reliable traits for *Cx. pipiens* included integument color (tan to brown) and scutal scale shape

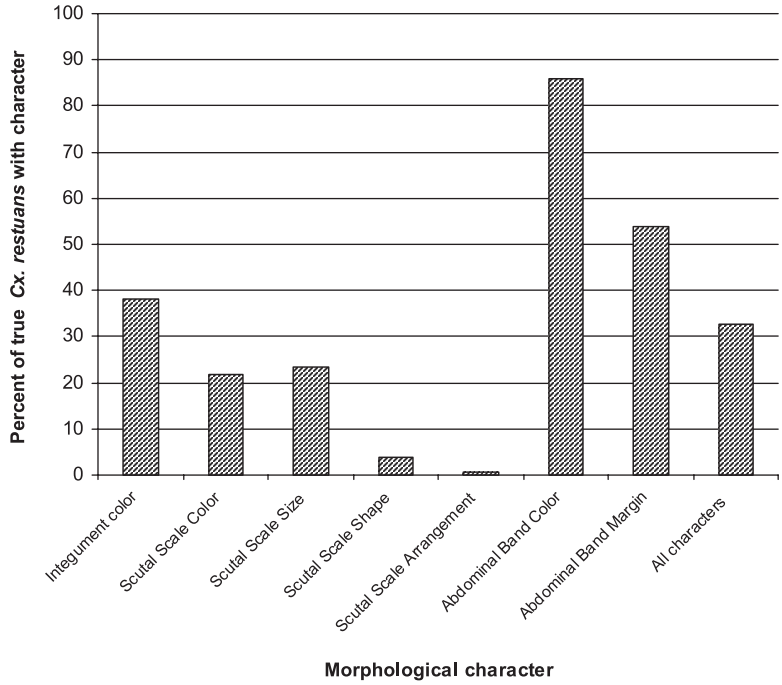


**Fig. 5.** Percentage of PCR-confirmed *Cx. restuans* that had the correct characters ($n$ = 121). The last column shows percentage of accuracy using all seven morphological characters.

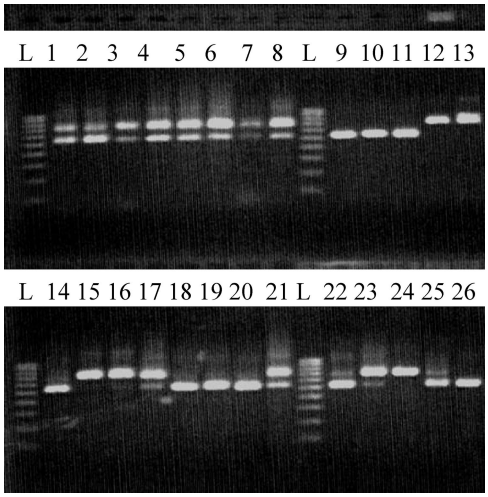


**Fig. 6.** A 1.5% agarose gel demonstrating amplification of mosquito samples with body parts from other species. Bands of 508 bp indicate *Cx. restuans*, and those at 698 bp indicate *Cx. pipiens*. Samples from the top row left are as follows (*Cx. restuans* = R, *Cx. pipiens* = P): L = genomic ladder; lane 1: 1R leg:1P leg; lane 2: 2R legs:1P leg; lane 3: 1R leg:3P legs; lane 4: 2R legs:2P legs; lane 5: 2R legs:3P legs; lane 6: 3R legs:2P legs; lane 7: 3R legs:3P legs; lane 8: 3R legs:3P legs; lane 9: 1R leg:0P legs; lane 10: 2R leg:0P legs; lane 11: 3R legs:0P legs; lane 12: 0R legs:1P leg; lane 13: 0R legs:2P legs. L = genomic ladder; lane 1: 1R leg:1P leg; lane 2: 2R legs:1P leg; lane 3: 1R leg:3P legs; lane 4: 2Rlegs:2P legs; lane 5: 2R legs:3P legs; lane 6: 3R legs:2P legs; lane 7: 3R legs:3P legs; lane 8: 3R legs:3P legs; lane 9: 1R leg:0P legs; lane 10: 2R legs:0P legs; lane 11: 3R legs:0P legs; lane 12: 0R legs:1P leg; lane 13: 0R legs:2P legs. Samples from the bottom row left are as follows: lane 14: R body:P head; lane 15: 1R legs:P head/thorax; lane 16: 2R legs:P head/thorax; lane 17: 3R legs:P head/thorax; lane 18: R head/thorax:1P leg; lane 19: R head/thorax:2P legs; lane 20: R head/thorax:3P legs; lane 21: R body:P body, lane 22; R head:2P legs; lane 23: 2R legs:P head; lane 24: R wing:P body; lane 25: 1R wing:1P wing; lane 26: R body:P wing.

(falcate) (73% of true *Cx. pipiens* specimens examined had these traits). The two most reliable traits for *Cx. restuans* were different from those of *Cx. pipiens.* Seventy-four percent of true *Cx. restuans* species had the *restuans*-type abdominal band color (white) and margin (broad, merging with white spot on lateral margin). Although care was taken to use the same light intensity to examine the color of each specimen in our study, changing light intensity and even the type of light used may alter perceived color.

A limitation of our study is that we did not directly compare male genitalia with other morphological traits. However, male genitalia characters are rarely used for field studies on mosquito ecology and vector-borne disease surveillance where trapping methods focus on collection of female mosquitoes. Furthermore, male genitalia are not used as discriminating characters for these two species in most mosquito keys for North America and the Northeast region (Darsie and Ward 1981, 2005; Means 1987; Andreadis et al. 2005).

Given the critical importance of these species as potential vectors of West Nile virus, it is important to understand the relative role of these species in vector-borne disease dynamics. A survey of the published literature using Biosis and Medline databases revealed that 37% of articles published since 1985 on the biology of *Cx. pipiens* and *Cx. restuans* used the same adult female morphological traits that we examined to identify *Cx. restuans* (21 of 57). Five of these articles were published in the past year alone (Anderson et al. 2006, Diuk-Wasser et al. 2006, Lampman et al. 2006, Molaei et al. 2006, Ngo et al. 2006). Additional articles fail to describe how these species were identified (Huhn et al. 2005, Gu and Novak 2006). All of these reports represent important field studies from the range of overlapping habitats for *Cx. pipiens* and *Cx. restuans* in the eastern United States.

Although there are clear and distinctive external characters to separate the larval stages of *Cx. restuans* and *Cx. pipiens* (Darsie and Ward 1981, Andreadis et al. 2004), characters are not as clear for the distinction of adults. The best morphological trait differentiating adult *Cx. restuans* was the presence of two faint white spots of scales on the thorax. Unfortunately, not all *Cx. restuans* emerge with these spots. In a recent study, 77% of field-collected and PCR-verified *Cx. restuans* from bird-baited exclusion traps had spots (Darbro and Harrington 2006) with significant variation by time of emergence. Furthermore, these small scales can be easily rubbed off in CDC light traps, gravid traps, and New Jersey-style traps.

Although molecular confirmation may be more expensive, and it may require additional training, we recommend that vector biologists confirm their morphological identification for these species with molecular data (Savage et al. 2006) or through examination of male genitalia (Carpenter and LaCasse 1955) if working with male specimens whenever possible. If these two methods are not possible due to time or financial constraints, we recommend using the two most reliable external morphology traits for each species as described above.

Unfortunately, care also must be taken with the molecular-based diagnostic approaches. Samples are susceptible to contamination by very small amounts of nonspecific DNA. In our study, a single leg of a nonspecific mosquito could contaminate the sample. For individual mosquitoes, contamination can easily be recognized by multiple bands on an agarose gel; however, for mosquito pools, it may be impossible to know how many mosquitoes in the pool represented another species.

Our conclusions are restricted to those mosquito species that cannot be clearly identified with morphology. We recognize the continuing and critical importance of traditional mosquito taxonomy in most field studies where species can be reliably identified. Although we focused our efforts on mosquitoes collected in New York state, we think that our study may have serious implications in other regions of North America where *Cx. restuans* and *Cx. pipiens* overlap (Darsie and Ward 2005) and where understanding

their biological differences is important. Not being able to differentiate these species obfuscates our understanding of their relative role and importance in transmission of arboviruses such as West Nile virus.

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