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PAPER

PATHOLOGY/BIOLOGY

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An Evaluation of Differentially Spliced Genes as Markers of Sex for Forensic Entomology*,†,‡

ABSTRACT: Blow flies (Calliphoridae) are important medically and economically and are commonly used in forensics as temporal markers in death investigations. While phenotypic traits in adult flies can be sexually dimorphic, sex identification in immatures is difficult. Consequently, little is known about how sex may result in developmental disparities among sexes even though there are indications that they may be important in some instances. Since genetic mechanisms for sex are well studied in model flies and species of agricultural and medical importance, we exploit the sex-specifically spliced genes *transformer* (*tra*) and *doublesex* (*dsx*) in the sex determination pathway to optimize a sex identification assay for immatures. Using known primer sets for *tra* and with a novel one for *dsx*, we develop PCR assays for identifying sex in four forensically relevant Calliphoridae species: *Lucilia sericata* (Meigen), *Lucilia cuprina* (Wiedemann), *Cochliomyia macellaria* (Fabricius), and *Chrysomya rufifacies* (Macquart) and evaluated their performance. Band detection rates were found to range from 71 to 100%, call rates ranged from 90 to 100%, and no error was found when bands could be called. Such information is informative for purposes of testimony and in preparation for development studies. The developed assays will assist in further differentiating sexually dimorphic differences in development of the Calliphoridae and aid in more accurately estimating insect age when age predictive markers (size, development time, molecular expression) are sexually dimorphic.

KEYWORDS: forensic entomology, sexual dimorphism, sex identification, genetics, Calliphoridae, blow flies, Diptera

The Calliphoridae (blow flies) are a diverse family of flies comprised of 54 species in North America, North of Mexico alone (1). These flies are of medical, economic, and forensic importance due to their close association with decomposition (2). Four species of interest are *Lucilia sericata* (Diptera: Calliphoridae) (Meigen), *Lucilia cuprina* (Diptera: Calliphoridae) (Wiedemann), *Chrysomya rufifacies* (Diptera: Calliphoridae) (Macquart), and *Cochliomyia macellaria* (Diptera: Calliphoridae) (Fabricius). All four species are of importance in veterinary entomology due to myiasis of livestock, which can be an economic burden in production agriculture and important in animal abuse investigations (3–5). In addition, *L. sericata* has importance in medical entomology where they are commonly used in maggot therapy for wound debridement (6,7). However, of most interest to this study, all four species are used in forensic investigations to supplement time of death estimations (8–10).

Various methods have been implemented to estimate the time of death in investigations, including classically understood markers such as rigor mortis and livor mortis (11). However, these processes only hold accurate for narrow windows of time relatively soon after death (12,13). Entomological evidence is useful in generating estimates of insect age. Though associated with various assumptions, estimates of insect age can provide information that speaks to investigatively important metrics such as minimum time since death/ minimum postmortem interval (min-PMI) (14).

Though insect development can provide accurate estimates, there remain limitations to the methodology (15). Some limitations, such as the ectothermic nature of insects, the impact of drugs and toxic substances, and cases of myiasis, are well studied (11,15–20). Sexual dimorphism, or the exhibition of differing characteristics between the two sexes, is common in the

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behavior, morphology, and physiology of most species with separate sexes (21). The impact of sexual dimorphism in the development of immature blow flies, and a way to account for this dimorphism in insect age estimates, is yet to be fully investigated. In phorids, current indications in the literature suggest that sexual dimorphism may lead to some inaccuracy and/or misinterpretation in PMI estimation (22). This is reinforced with the observation of different growth curves between male and female blow flies which may lead to variation in minPMI estimations for forensic investigations (23); however, not all sexual dimorphism results in development time differences between males and females (24). Species without development time differences between the sexes would not be expected to impact casework results; however, other species may have large enough differences in development times between sexes to have an impact on minPMI estimates for casework. In beetles, the use of size and sex is useful in improving age estimates of forensically important species in at least one documented instance (25,26). While the impact of sexual dimorphism in development time is acknowledged (22,23,27), methods which have quantitatively evaluated the reliability to identify sex of immature Calliphoridae are lacking. In order to apply information from sex-specific datasets for interpretation of evidence, error rates for identification of sex are necessary. Such information is critical to address Daubert standards related to forensic entomology research and in planning studies of sexual dimorphism in immatures.

Sex determination during development can occur due to sex chromosome complement (XY vs XX), environmental conditions (28), or even the ploidy level of an organism (29,30). While differences in sex chromosome size may allow for sex identification in some species by flow cytometry, such as with *L. sericata* (31), not all species have sexes with distinguishable genome sizes. Additionally, this method requires specific storage conditions which may not be met in all cases and would require samples to have been frozen immediately upon collection or sampled immediately after death. Successful identification of insect age, through gene expression (18,32–34), suggests the use of sex-specific gene expression as a route to immature insect sex identification. In a variety of species, there is evidence for many genes across the genome with sex-biased expression and many genes with sex-specific isoforms due to differential splicing (35–37). These sex-specific isoforms can contribute to sex-specific phenotypes (21), such as pigmentation in *Drosophila* (Diptera: Drosophilidae) (Fallen), horn volume and body mass in bighorn sheep, facial adornments in primate species, and many other traits are classic examples of sexual dimorphism (38–40).

In flies, sex chromosomes carry the master switch for sex determination (41–43), which can then lead to gene expression differences across the genome which determine sex-specific phenotypes. There is strong homology between *Drosophila* and most Calliphoridae in the downstream portion of their sex determination pathways (44). However, the top of the hierarchy between *D. melanogaster* and calliphorids is not conserved (45). In the shared component of the system, in females, the *transformer* (*tra*) gene is spliced to its female isoform (tra^F) (46), which alongside *transformer 2* (*tra 2*), regulates the splicing to either *doublesex* (*dsx*) or *fruitless* (*fru*) female-specific isoforms (47–49). In males, there is a suppression of splicing and the long form of male *tra* transcript (tra^M), which is nonfunctional, and results in male-specific splicing of *dsx* and *fru* (50). *Sex lethal* (*Sxl*) and an X signal element (XSE) are not expected to dictate blow fly sex determination, rather a dominant male determining (M factor) is expected to drive splicing of tra^F and tra^M transcripts, which are determined through the first intron splicing site (44). The presence of tra^M inhibits *tra* RNA splicing while maternal TRA contributes to initiation of tra^F splicing (45). After *tra* splicing, the expression patterns are homologous to that in *Drosophila* (45). This pathway is seen to function generally as expected in *L. sericata, L. cuprina,* and *Co. macellaria* (44,45,51,52) (Fig. 1).

Ch. rufifacies differs from other calliphorid species in that it has monogenic sex determination, meaning females produce single-sex offspring (53,54). In this species, *tra* seems to be nonfunctional or missing from the transcriptome (55). However, the species does exhibit dimorphic expression of *dsx;* thus, this locus is currently the most likely target for differentiating between sexes of immatures.

Here, we optimize PCR assays for the identification of sex in immature stages of four species of Calliphoridae of forensic importance. Through the optimization of these sex identification assays, a method was developed to account for sexually dimorphic markers of age in male and female blow flies in forensic applications. One of these assays is novel. Though this type of work has previously been accomplished in some species for alternative applications (44), this is some of the first work for sex determination assays for forensic science applications. Additionally, this research focused heavily on use in immature samples that are of considerable forensic interest. This study also determines detection rates, call rates, and error rates for these methods—statistics which are not a focus in previous studies (44), but which are important for forensic applications due to expectations of quantification of error in forensic assays and is important information for colleagues that are planning dimorphism research with these markers.

Materials and Methods

Adult flies were frozen at −80°C and then morphologically identified before extraction (1). Vouchers for identification can be found at the Texas A&M University Insect Collection (TAMUIC)—*L. sericata*: voucher #726 (immature), #747 (adult), *L. cuprina*: voucher #736, *Co. macellaria*: voucher #731, *Ch. rufifacies*: voucher #730. TRI Reagent® (Molecular Research Center, Inc., Cincinnati, Ohio) was used to extract the RNA. Each sample (i.e., a single larva or pupa) was extracted according to manufacturer's protocol. Quantification of the RNA was performed with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc.®, Wilmington, Delaware). Samples that contained greater than 1000 ng/μL of RNA were diluted with DNase/RNase/Nucleotide-free H_2O.

Digestion of DNA and purification of the RNA before conversion to cDNA were performed using amplification grade deoxyribonuclease I (DNase I) (Invitrogen™). Two μ*g* of RNA was added into a 0.5 mL RNA-free tube on ice along with 2 μL DNase I. The final volume was brought to 10 μL with DNase/RNase/Nucleotide-free H_2O. The tube was incubated at room temperature for 15 min, and then, 1 μL of ethylenediaminetetraacetic acid (EDTA) was added to the solution. The samples were heated for 10 min at 65°C on a thermal cycler.

The RNA was converted to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, California). Kit components were thawed on ice. A master mix was prepared with 2 μL 10× reverse transcriptase (RT) buffer, 0.8 μL 25× deoxyribonucleotide (dNTP) Mix (100 mM), 2 μL 10× RT Random Primers, 1 μL MultiScribe Reverse Transcriptase, and 4.2 μL DNase/RNase/Nucleotide-free H_2O for a

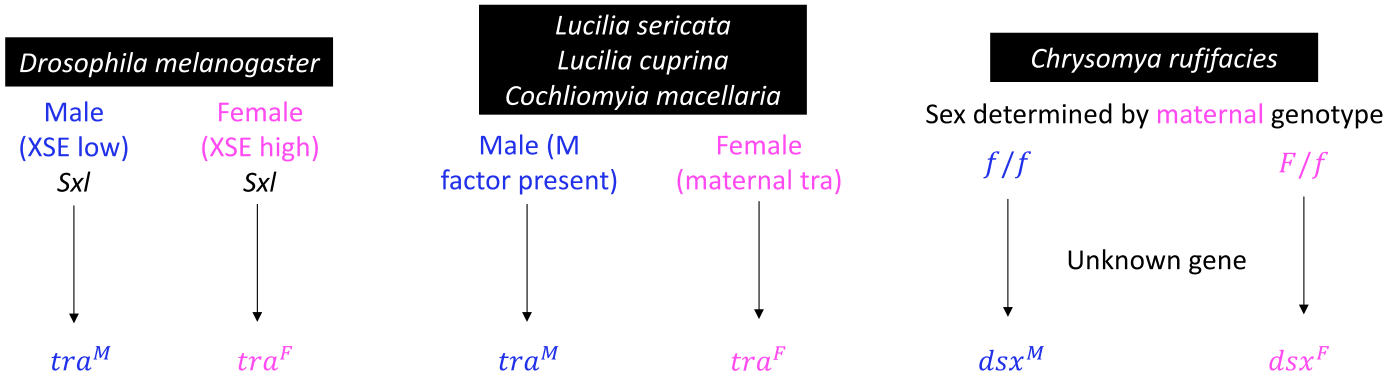

FIG. 1—*A comparison of the sex determination mechanisms in* Drosophila melanogaster, Lucilia sericata, L. cuprina, Cochliomyia macellaria, *and* Chrysomya rufifacies. *In* Drosophila, *X-signal elements (XSE) determine the splicing of transformer (tra). Similarly, in blow flies, we see homology in their sex determination mechanism, where rather than XSE, the presence or absence of an M factor will determine tra splicing. One blow fly that differs from this conserved mechanism is* Ch. rufifacies *where the gene that regulates sex determination is unknown; however, doublesex (dsx) is expected to be conserved within this species (45).*

total reaction volume of 10 µL. The 10 µL of master mix was added to 10 µL of the RNA sample in a 0.5 mL PCR strip tube on ice. The solution was mixed by vortexing to ensure mixture and then briefly spun down to eliminate air bubbles. The sample was converted to cDNA according to manufacturer protocols along with a –RT control to rule out DNA contamination in the sample. After removal from the thermal cycler, the samples were kept at −20°C.

Primers for optimization within each species were either gathered from previous research (*L. sericata, L. cuprina, Co. macellaria*) (44) or designed using published transcriptome data (Appendix S2) and primer-BLAST (*Ch. rufifacies*) (56). Additionally, primers were developed using *L. cuprina* for use in both *Lucilia* species. The *Ch. rufifacies dsx* assay was distinct in that there is a shared sequence followed by male- and female-specific sequences that needed to be targeted for primer design in each sex, meaning two PCRs are done per sample instead of one as done with the *tra* assays. All primers were used in a 10 mM dilution with DNase/RNase/Nucleotide-free H_2O. Detailed below is the primer design for each species of interest for this research. This primer set was then optimized through temperature gradient polymerase chain reaction (PCR) for an assay that can determine sex in larvae and pupae as well as adults, for *L. sericata, L. cuprina, Co. macellaria,* and *Ch. rufifacies*.

All PCR analyses were completed using Thermo Scientific™ PCR Master Mix (Thermo Scientific™, Waltham, Massachusetts). This PCR master mix was composed of 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM magnesium chloride, and 0.4 mM dNTP. The PCR reactions, unless otherwise stated, were prepared using 15 µL PCR master mix (2×), 10 µL DNase/RNase/Nucleotide-free H_2O, 1.5 µL forward primer, 1.5 µL reverse primer, and 2 µL of cDNA. Primers for each reaction correspond to those in Table 1. For gradient PCR, a PCR reaction prepared as stated above was used for each sample under the conditions stated in Table 2. The optimal annealing temperature for each species *L. cuprina*, *L. sericata*, *Co. macellaria,* and *Ch. rufifacies* is 48.9, 48.9, 57.7, and 54.0°C, respectively. Resultant PCR products were separated by running for approximately 1 h on a 1% agarose gel at 120v and then visualized under UV light. Presence of cDNA for each sample was validated with the *rp49* housekeeper gene using qPCR analysis (32,57) on a traditional benchtop RT-PCR machine (BioRad CFX96 Real-Time System with C1000 Thermal Cycler, Bio-Rad Laboratories, Hercules, California). Each qPCR plate was run with a positive, negative, and no-template control. Each sample reaction contained 5 µL SSoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.), 2 µL primer pair, 1 µL DNase/RNase/Nucleotide-free H_2O, and 2 µL cDNA. These reactions were run under the following conditions: 40 cycles of denaturation at 94°C for 45 sec and annealing/extension at 72°C for 60 sec, followed by a 65–95°C melt curve at increments of 0.5°C.

The highest annealing temperature with splicing products present from gradient PCR analysis (Appendix S1) was used as the temperature for error rate determination in each species. To determine the error rate of the assays created, a double-blind testing of known sex determination was performed on 80 samples, 20 for each species. For error rate determination, *L. cuprina*, *L. sericata*, *Co. macellaria,* and *Ch. rufifacies* used annealing temperatures 48.9, 48.9, 57.7, and 54.0°C, respectively.

TABLE 1—*The forward and reverse primer sequence, transcriptome assembly or previous primer sequence used, and expected splicing product size for males and females of each species.*

Species	Forward Primer Sequence	Reverse Primer Sequence	Sequence Used	Expected Splice Product (base pairs)
Lucilia sericata, L. cuprina	5'-ATT TAA AAT TCA ACA ATC CAT ACC C-3'	5'-TCT AAA TTA TTA GTA TCA CGA GCA T-3'	Li et al. 2013 (JX315620), Sze 2012	F-193 bp M-511 bp
Cochliomyia macellaria	5'-ATA CCA AGT GGT TCG GTG AAA AGA GGT C –3'	5'-GGT TTT AGT TTT ACC GCT TGT ATG GTG TTC –3'	Li et al. 2013 (JX315619)	F-204 bp M-502 bp
Chrysomya rufifacies Male	5'-TGT GAT GAA ACA ACC AAA CG-3'	5'-ACC ACC TAT ACT ACT ACC CG-3'	Sze et al. 2017	F-None M-285 bp
Ch. rufifacies Female	5'-GCC ATG TTC CTG CTG CTC TA-3'	5'-CAC ATT GTC GGG TGG CAC AA-3'	Sze et al. 2017	F-684 bp M-None

TABLE 2—*The thermal cycle parameters used for the assays.*

	Initial Incubation	Denature	Anneal	Extend	Final Extension	Final Step
Temperature (°C)	95	95	Varies between species	72	72	4
Time	3 min	30 sec	30 sec	1 min	4 min	∞

The denaturation, annealing and extension steps were repeated for 35 cycles. The various species have differing annealing temperatures for their respective assays—*L. sericata* and *L. cuprina* (48.0°C), *Co. macellaria* (57.7°C), and *Ch. rufifacies* (54.0°C).

For sequencing, PCR products from the *dsx* assay were treated with the ExoSAP-IT™ reagent (Applied Biosystems™, Santa Clara, CA, USA) following the manufacturer's protocol. To identify the three bands produced by the PCR reactions, Sanger sequencing was performed on two samples of 700 base pair bands (MN178928), two samples of 400 base pair bands (MN178929), and two samples of 300 base pair bands (MN178930) at the Laboratory for Genome Technology at the Institute for Plant Genomics and Biotechnology at Texas A&M University. Sanger sequencing reads were assembled for each band using the sangeranalyseR (58) (https://github.com/roblanf/sangeranalyseR) and were deposited in NCBI (Accession numbers: MN178928-MN178930).

Results

cDNA Quality

All cDNA samples were analyzed with qPCR to determine whether samples were likely to produce a result and to ensure no presence of genomic DNA by confirming a lack of amplification in -RT controls. The housekeeper gene *rp49* was used to perform this check which has a known melt temperature of approximately 78.5°C (57). All -RT samples did not amplify, indicating a lack of the presence of genomic DNA, while all RT + samples yielded PCR products with a single melt temperature at approximately 78.5°C.

Optimized Parameters

The final optimized parameters for each of the four assays were determined using gradient PCR. An annealing temperature of 48.9°C is used for *L. cuprina* and *L. sericata*, 57.7°C is used for *Co. macellaria*, and 54.0°C is used for *Ch. rufifacies*. These optimized annealing temperatures are used for the error rate determination. For error rate determination, the expected splicing products are ~200 bp for female and ~500 bp for male *L. sericata and L. cuprina*, ~200 bp for female and ~500 bp for male *Co. macellaria*, and ~300 bp for male and ~700 bp for female *Ch. rufifacies* (Fig. 2*A*). An additional ~350 bp product is seen in male *Co. macellaria,* and though this band is not expected, its presence is not surprising (Fig. 2*B*). Major and minor bands for the *tra* gene have previously been observed in other calliphorid species (44). In the known samples, the 500 bp band is always present in males, while the 350 bp band is not. Additional samples run thus far with the assays can be seen in Table 4.

Detection, Call, and Error Rates in Adult Specimens

The performance of all assays with adult samples was evaluated by assessing detection rates (successful PCR reactions), call rates (successful PCR reactions that could be unambiguously assigned to a sex), and error rates (when called samples were incorrectly predicted) when predicting sex with molecular markers. For all error rate determinations, twenty adult samples and a negative control were tested per species to determine the accuracy of the sex identification assay. All negative controls did not amplify. For *L. sericata*, a ~200 bp product was used as a basis for visualization in female samples and a ~500 bp product for male samples. Of the 19 of 20 PCR samples that yielded a result, all 19 were assigned to the appropriate sex with a call rate of 100% and a detection rate of 95% (Table 3). For *L. cuprina*, a ~200 bp

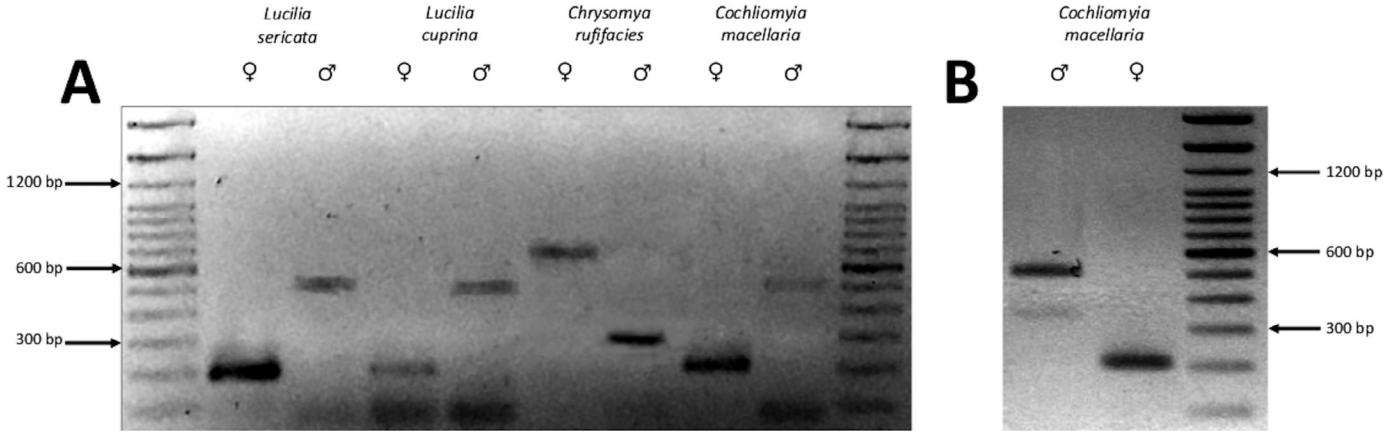

FIG. 2—*(A) In this gel image, lane 1 and 10 is the ladder used to identify male and female samples by their expected transcript size. Lane 2 and 3 represent* Lucilia sericata *female and male, respectively, with females showing a product at ~200 bp and males showing a product at ~500 bp. Lane 4 and 5 represent* L. cuprina *female and male samples, respectively, with females showing a product at ~200 bp and males showing a product at ~500 bp. Lane 6 and 7 represent* Chrysomya rufifacies *female and male, respectively, with females showing a product at ~700 bp and males showing products at ~300 bp. Lane 8 and 9 represent* Cochliomyia macellaria *female and male products, respectively, with females showing a product at ~200 bp and males showing products at ~500 bp. (B) In this gel image, lane 1 and 2 represent* Co. macellaria *male and female products, respectively. Males show a product at ~350 and ~500 bp, and females show a product at ~200 bp. Lane 3 is the ladder used to identify male and female samples by their expected transcript size.*

TABLE 3—*Error rate determinations for sex identification of the species* Lucilia sericata*,* L. cuprina*,* Cochliomyia macellaria*, and* Chrysomya rufifacies *with 20 adult samples for each species.*

	Locus	Samples with Bands Present	Samples with No Bands	Number of Samples with Unambiguous Products	Number Correct Predictions	Call Rate	Detection Rate	Total
L. cuprina	*tra*	18	2	18	18	100%	90%	20
L. sericata	*tra*	19	1	19	19	100%	95%	20
Co. macellaria	*tra*	19	1	19	19	100%	95%	20
Ch. rufifacies	*dsx*	20	0	18	18	90%	100%	20

Of the 20 samples, those that could be identified on gel electrophoresis were compared with known sex that was previously identified before analysis. When bands were detected, sometimes they were ambiguous. The number called is those bands that were unambiguous. The call rate is those unambiguous bands that were called while the detection rate is the percentage of bands present. Additionally, from this, the call rate and detection rate are calculated for each of the three species.

product was used as a basis for visualization in female samples and a ~500 bp product for male samples. Of the 18 of 20 PCR samples that yielded a result, all 18 were assigned to the appropriate sex with a call rate of 100% and a detection rate of 90% (Table 3). For *Co. macellaria*, a ~200 bp product was used as a basis for visualization in female samples, and a ~350 and ~500 bp product were used as the basis for visualization in male samples. Of the 19 of 20 PCR samples that yielded a result, all 19 were assigned to the appropriate sex with a call rate of 100% and a detection rate of 95% (Table 3). One sample did not contain any bands. For *Ch. rufifacies*, a ~700 bp product was used as a basis for visualization in female samples, and a ~300 bp product was used as the basis for visualization in male samples. Of the 20 PCR samples that yielded a result, 18 were assigned to the appropriate sex with a call rate of 90% and a detection rate of 100% (Table 3). The other two samples were not called because the samples produced bands for both male and female products.

Performance with Immature Specimens

After determining error rates in adult specimens, cDNAs derived from immature blow flies were assessed (Table 4). Specimens referred to as "pupae" are intrapuparial but for simplicity sake are designated as such (59). Generally, the assays performed as expected in immature samples. However, there were key differences. First, a ~400 bp product was identified in the *Ch. rufifacies* assay in 20 of 344 immature individuals. The 400 bp band has only been visualized in the pupal and adult stage but shows up both with the 700 bp band and with the 300 bp band. The band was visible both in a gel electrophoresis imaging post a 30 uL PCR reaction run at 35 cycles and after a 60 uL PCR reaction run at 40 cycles; however, only 4 of 20 samples maintained both bands after the 60 uL PCR reaction. This unexpected band was considered insufficient to call a sex, as justified by sequencing results discussed below. Second, PCR volumes needed to be adjusted for some samples. If a 30 μL PCR reaction was not sufficient to observe or call bands, those samples were reamplified with a 60 μL PCR reaction and the PCR reaction cycles were increased to 40 cycles. Overall, after accounting for the distinctions noted above, 50 (*Ch. rufifacies* feeding larvae)-100 (*L. sericata* pupae) % of specimens could be assigned to a sex.

Sequencing of *Ch. rufifacies* PCR Products

Sanger sequencing was completed to confirm products for *dsx* in female *Ch. rufifacies*. Consensus sequences were

TABLE 4—*All identifications that have been completed with the four sex identification assays and the life stages that each of the samples comes from.*

Species	Stage	Female	Male	Un-determined	No bands	Total	Detection Rate	Sample Age in Hours (25°C)
Lucilia cuprina	Larvae	0	0	0	0	0	N/A	–
	Pupae	41	35	0	3	79	96%	128–385
	Adult	9	9	0	2	20	90%	–
	Total	50	44	0	5	99	95%	–
L. sericata	Feeding 3rd	20	5	0	0	25	100%	120–132
	Postfeeding 3rd	13	11	0	1	25	96%	144–156
	Early Pupae	3	9	0	0	12	100%	168
	Mid-Pupae	18	6	0	0	24	100%	212–324
	Late Pupae	11	1	0	0	12	100%	372
	Adult	10	9	0	1	20	95%	–
	Total	75	41	0	2	118	99%	–
Cochliomyia macellaria	Larvae	0	0	0	0	0	N/A	–
	Pupae	29	18	0	4	51	92%	189.5–289.5
	Adult	10	9	0	1	20	95%	–
	Total	39	27	0	5	71	93%	–
Chrysomya rufifacies	Feeding 3rd	2	6	0	8	16	50%	119.5–120
	Postfeeding 3rd	9	13	0	2	24	92%	149–190
	Early Pupae	38	38	0	46	122	62%%	214–238
	Mid Pupae	39	43	1*	16	99	84%	262–286.5
	Late Pupae	31	37	3*	17	88	81%	310–358
	Adult	8	11	2*	0	20	90%	–
	Total	135	129	6	95	364	74%	–

Additionally, information of the detection rate for each life stage and the hours for pupae and larvae show the hours since eggs were laid (oviposition). All specimens in this study were raised at 25°C.

*Indicates those samples with the spurious 400 bp band.

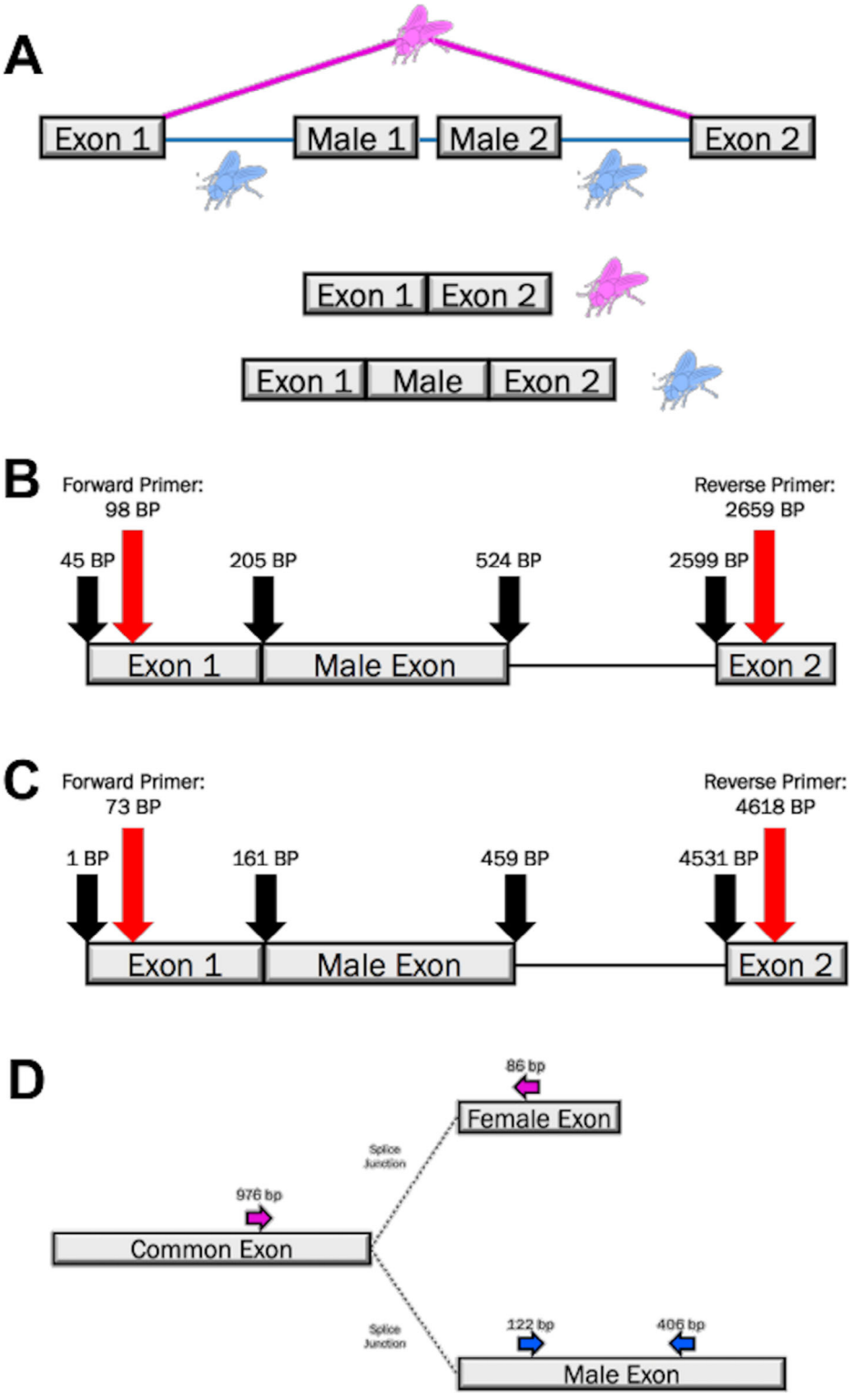

FIG. 3—*In this figure, the expected splice variants from Calliphoridae are shown. (A) Females will have splice products that contain exon 1 and exon 2. On the other hand, males will have splice products that contain exon 1, exon 2, and some variation of a male exon. This creates a male splice product that is longer than the female splice product and allows for a way to identify sex in a molecular approach. This expected splice product is seen in the three species* Lucilia sericata, L. cuprina, *and* Cochliomyia macellaria *as the tra gene is targeted for these assays. (B) This shows the expected splice product size from* L. sericata *and* L. cuprina *based on the primers used and the known gene sequence. This is based on NCBI gene sequence JX315620.1, and it is expected* L. cuprina *will have a similar size band as these species are similar in their sex determination mechanism. (C) This shows the expected splice product from* Co. macellaria *based on the primers used and the known gene sequence. This is based on NCBI gene sequence JX315619.1. (D)* Chrysomya rufifacies *is unique in the splice products it produces as dsx rather than tra is being targeted for this assay.* Ch. rufifacies *shares a common exon between males and females and sex-specific exons for each sex. This shows the expected splice products from* Ch. rufifacies *for males and females based on the primers used and the known transcriptomic sequence. As sequences were gathered from the transcriptome, the length of introns and placement of exons is not known.*

BLASTed to determine what gene/product they mapped back to. The expected 700 bp band designed from a published transcriptome matches best to a female isoform of *dsx* of *L. cuprina* (percent identity: 82.41%). Upon sequencing, the spurious 400 bp band is not *dsx* but rather matches best to 28S rRNA of *Co. macellaria* (percent identity: 98.80%). The expected 300 bp band designed from a published transcriptome matches best to male isoform of *dsx* in *L. cuprina* (percent identity: 82.13%).

Discussion

Our results suggest that targeting the conserved *tra* or *dsx* genes within the four calliphorid species evaluated is a viable method for sex identification that can be used in forensic investigations with expectation of low error rates, but with some degree of dropout from failed reactions and ambiguous results. The gene products of *tra* and *dsx* are important for the sex identification assay, as they are differentially spliced and yield sex-specific splicing products. Though the basic capability was already established ([24, 44]; Fig. 3), we provide a new primer set for the problematic species *Ch. rufifacies* and an assessment of performance of such primers in immature specimens with an eye toward forensic application, identifying practical issues with detection in some immature samples.

In our research, we chose to optimize the assay for use in forensic applications and with immature specimens. The use of immature specimens resulted in low levels of expression for the genes analyzed in these specimens. This necessitated large volume PCRs to gain an adequate signal for gel visualization of these samples. For example, in the species *L. sericata*, of the 98 total larvae and pupae samples, 21 samples (10 larval, 11 pupal) showed no bands after a 30 μL PCR reaction. Additionally, most samples were female that did not amplify (out of the 21 samples-16 females, 4 males, and 1 with unknown sex). When again tested using a 60 μL PCR reaction, only one of these 21 samples showed no bands. This may be due to species specific effects, or it may be caused by the stage of the sample. Future work could explore additional options for improving upon this strategy for detecting rare transcripts in some developmental stages. Knowledge of detection rate is important for researchers developing projects, as they need to know the expected number of failed assays with immature specimens when they develop sex-specific reference data.

Measuring detection rates, call rates, and error rates are key to the use of this method in forensic investigations as this sort of information is expected in forensic applications (60,61). The most important consideration with these assays appears to be the detection and call rates. However, once successful calls are made, error rates do not appear to have been large enough to observe with the reported sample sizes. Of most concern in this study was the call rate in *Ch. rufifacies*, which is lower than other species. Detection rates appear lower in early larval and intrapuparial samples, as increasing PCR volumes seems to rescue some failed PCRs for this species. This is indicative of low *dsx* expression early in development. Additionally, call rates seem to be affected with late intrapuparial samples (Table 4). The call rate may be explained by leaky splicing in the *dsx* gene, which has been observed in *Drosophila* species in qPCR (62), microarray (63), RNA-seq (64–67), and *in situ* (68). Leaky splicing results from incomplete splicing of mRNA to the isoforms canonically associated with the appropriate sex. Thus, while males may express mostly dsx^{M}, some lineages can express relatively small amounts of dsx^{F}. In immature samples, some tissues seem to express both isoforms of the gene (68). Thus, in cases of leaky splicing in *dsx*, while higher concentrations of the appropriate product are found in the correct sex, there are detectable levels of product typically attributed to the other sex (62–64). If there is leaky splicing in a sample, this will lead to two fragments present for a sample in our assay and the potential to fail to call a sex in a sample. Leaky splicing, however, will occur in low numbers as strong leakiness of this gene into an inappropriate transcript would have harmful fitness effects. Additionally, as the number of PCR cycles increases, the ability to differentiate concentration in bands becomes more challenging. There will likely be a tradeoff in high numbers of cycles to detect a *dsx* product and ambiguity in results from leaky splicing. Thus, call rate and detection rate appear to be issues with distinct developmental groups, which may present specific challenges when attempting to predict their sex.

A unique attribute of sex identification in *Ch. rufifacies* samples is the presence of two bands of differing product lengths in female—either a single band at 700 base pairs (which appears to be dsx^{F} by our sequencing results) or one at 700 base pairs with an additional band at 400 base pairs. This smaller band is rarely seen and only in pupal and adult samples. When it is visualized, it is never more abundant than the 700 base pair band. It is unclear whether this spurious band is relevant given the distinct sex determination system; however, it is found to BLAST to *Co. macellaria* 28S rDNA. There is no evidence that this sequence BLAST hit to *Co. macellaria* is due to contamination as reverse transcriptase and PCR controls were negative and species were extracted separately. These results are likely because there is no complete *Ch. rufifacies* 28S sequence present in BLAST and 28S is highly conserved among the two species. Future optimization of this assay for work in *Ch. rufifacies* could focus on avoiding the production of this product or using methods such as RACE to target larger sequences for design of primers.

In summary, these assays will be a tool for identifying immature insects of forensic relevance. As previously mentioned, we know that there are developmental differences that occur in some blow fly species and there is not currently a way to confidently identify assess sex in immature forms for all forensically important taxa. Future work will include investigating ambiguous or unexpected banding patterns in immatures and optimization of sex identification in developmental stages where expression occurs at low levels. Utility of this assay in forensic investigations will be possible given the determination of low error rates. Ultimately, this type of assay will allow investigators to identify the sex of immature samples for forensic investigations and then to use sex-specific development information to determine whether sex is relevant in their particular situations (24) and in their calculations whether sex is considered to produce more accurate and/or precise estimates. As more and more work in forensic entomology is associated with molecular phenotypes, these sorts of assays may be particularly important as many molecular phenotypes have been demonstrated to be sexually dimorphic in flies (40,69,70). Thus, we provide evidence of reliability in transcript-based estimates of blow fly sex in adult and immature specimens. We demonstrate rates of expected dropout that are relevant to reference data set development and forensic applications. Finally, we have provided a novel assay for one blow fly species with an odd sex determination mechanism.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Methods for gradient PCR.

Appendix S2. *Chrysomya rufifacies* exon sequences for the doubles (dsx) gene.