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Author(s) :Joel F. Gibson, Scott Kelso, Morgan D. Jackson, Joel H. Kits, Gil F. G. Miranda, and Jeffrey H. Skevington

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# Diptera-Specific Polymerase Chain Reaction Amplification Primers of Use in Molecular Phylogenetic Research

JOEL F. GIBSON,<sup>1,2,3</sup> SCOTT KELSO,<sup>1</sup> MORGAN D. JACKSON,<sup>4</sup> JOEL H. KITS,<sup>4</sup>  
GIL F. G. MIRANDA,<sup>4</sup> AND JEFFREY H. SKEVINGTON<sup>1,2</sup>

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**ABSTRACT** DNA sequence data from a variety of mitochondrial and nuclear gene regions are significant components of phylogenetic research in entomology. Polymerase chain reaction (PCR) amplification primers for many gene regions have been developed that are specific to a range of dipteran groups. Here, we review the existing Diptera-specific PCR amplification primers that have been published for 11 mitochondrial and nuclear gene regions: 12S small ribosomal subunit, cytochrome *b*, cytochrome oxidase *c* subunit I, 28S ribosomal RNA, alanyl-tRNA synthetase, the carbamoyl phosphate synthase region of CAD, elongation factor-1 $\alpha$ , 6-phosphogluconate dehydrogenase, triose phosphate isomerase, *white*, and *wingless*. We also have designed in total 94 new PCR amplification primers for use in these same gene regions. Our new primers have been developed and tested using our DNA sequence database of >1,600 specimens representing 40 families of Diptera. All of the past and newly developed primer sequences are presented in tables, and their locations are shown on gene maps. This combined data will facilitate future molecular phylogenetic research within Diptera.

**KEY WORDS** gene maps, phylogenetics, mitochondrial DNA, nuclear DNA, ribosomal DNA

The most recent review of published, DNA sequence-based, phylogenetic studies of insects (Caterino et al. 2000) lists a large number of studies using a broad range of target taxa and gene regions. In the time since the publication of this review, many more papers have been published that use many different gene regions to study relationships between a variety of taxa. Although some of these studies include the development of new, taxon-specific polymerase chain reaction (PCR) amplification primers, many rely on existing, published primers. These existing primers, however, may not be appropriate for the taxa being investigated and may lead to inefficiency or sequencing failure.

Generally, the primers used to generate DNA sequence data were developed for use in groups of insects other than those that are the focus of the new study. For these primers to be of use in sequencing taxa that have never before been sequenced, universal primers are a necessity. Universality takes the form of oligonucleotide degeneracy or an acceptable level of oligonucleotide mismatch. Both situations can make the amplification and sequencing of target gene regions extremely difficult, if not impossible. Degenerate primers can produce nonspecific amplification,

multiplex PCR product, and the necessity of isolating the desired PCR product before sequencing. Although new techniques exist (Ma and DiFazio 2008, Gibson et al. 2010a) to facilitate the isolation of desired PCR products from multiplex PCR products, these methods require additional time and money. Also, when degeneracy is included in a primer sequence, each different possible version of the primer sequence is produced and included in the manufactured product. This can lead to an exponential increase in the number of primer sequences present in the PCR reaction. For example, a manufactured primer oligonucleotide sequence containing four N's and three Y's would actually consist of 2,048 different nucleotide sequences, only one of which would match the genomic template DNA sequence. This leads not only to a greatly reduced concentration of the correctly matching primer but also a disruption in reaction kinetics as genomic template primer locations are blocked by poorly matching versions of the primer. Primers with less degeneracy, but developed for distantly related groups, often lead to sufficient nucleotide mismatch to result in amplification failure.

Another consideration is the condition of the specimen from which DNA is being extracted. In molecular phylogenetic research, the specimens being used may not have been prepared or stored under optimal conditions. These conditions may have led to degradation and fragmentation of genomic DNA. In these instances, a number of alternate primer pairs may be necessary to amplify and sequence the target gene region in smaller segments.

<sup>1</sup> Agriculture and Agri-Food Canada, Canadian National Collection of Insects, Arachnids and Nematodes, 960 Carling Ave., Ottawa, ON K1A 0C6, Canada.

<sup>2</sup> Department of Biology, 209 Nesbitt Bldg., Carleton University, 1125 Colonel By Dr., Ottawa, ON K1S 5B6, Canada.

<sup>3</sup> Corresponding author, e-mail: joel.gibson@agr.gc.ca.

<sup>4</sup> School of Environmental Sciences, 1216 Edmund C. Bovey Bldg., University of Guelph, Guelph, ON N1G 2W1, Canada.

**Table 1.** Number of specimens of each Diptera family included in data set used to create new PCR amplification primers for each gene region

Family	Gene region										
	12S	Cytb	COI	28S	AATS	CAD	EF-1 $\alpha$	PGD	TPI	<i>white</i>	<i>wingless</i>
Atelestidae	1	0	1	1	0	1	0	0	0	0	0
Bombyliidae	1	0	1	1	0	1	0	0	0	0	0
Brachyostomatidae	1	0	1	1	1	1	1	1	1	1	1
Conopidae	71	65	69	71	66	11	6	8	8	9	10
Curtonotidae	1	0	1	1	0	1	0	0	0	0	0
Cypselosomatidae	1	0	1	1	0	1	0	0	0	0	0
Diopsidae	4	0	3	4	3	4	4	3	4	2	4
Dolichopodidae	1	0	1	1	0	1	0	0	0	0	0
Drosophilidae	1	1	1	1	1	1	1	1	1	1	1
Empididae	1	0	1	1	0	1	0	0	0	0	0
Heleomyzidae	1	1	1	1	1	0	0	0	0	0	0
Hybotidae	1	0	1	1	0	1	0	0	0	0	0
Ironomyiidae	1	0	1	1	0	1	0	0	0	0	0
Lauxaniidae	2	2	3	2	2	2	2	1	1	2	2
Lonchopteriidae	2	1	2	2	1	2	1	1	1	1	1
Marginidae	1	0	1	1	0	1	0	0	0	0	0
Megamerinidae	1	0	1	1	1	1	1	1	1	1	1
Micropezidae	46	1	38	13	5	42	11	5	5	4	35
Muscidae	2	1	2	2	1	2	0	0	1	1	1
Neriidae	1	0	1	1	1	1	1	1	1	1	1
Opetiidae	1	0	1	1	0	1	0	0	0	0	0
Pallopteriidae	1	1	1	1	1	1	1	1	0	1	1
Phoridae	3	1	3	3	1	3	1	1	1	0	1
Pipunculidae	88	42	80	2	44	78	1	1	1	1	1
Platypzeidae	2	1	2	2	1	2	1	1	1	0	1
Platystomatidae	1	1	1	1	1	1	1	1	1	1	1
Psilidae	2	0	2	2	2	2	2	1	1	2	2
Pyrgotidae	1	1	1	1	0	1	1	1	0	1	1
Richardiidae	1	0	1	1	0	1	0	0	0	0	0
Sciadoceridae	1	0	1	1	0	1	0	0	0	0	0
Sciomyzidae	1	0	1	1	0	1	0	0	0	0	0
Somatiidae	1	0	1	1	1	1	1	1	1	1	1
Sphaeroceridae	26	22	24	23	18	2	1	0	1	1	1
Strongylophthalmyiidae	2	1	2	2	2	2	2	1	2	2	2
Syringogastridae	1	0	0	1	1	1	1	1	1	1	1
Syrphidae	44	39	44	40	32	39	1	1	1	1	1
Tachinidae	1	0	1	1	0	1	0	0	0	0	0
Tanypezidae	1	0	1	1	1	1	1	1	1	1	1
Tephritidae	1	1	1	1	1	1	1	1	0	0	0
Therevidae	1	0	1	1	0	1	0	0	0	0	0
Total no. specimens included	319	182	298	193	189	215	44	35	36	36	72
Total no. families included	40	17	39	40	24	39	23	22	21	21	23

Due to the potential problems introduced by overly degenerate or mismatched primers and the necessity for primers located throughout the length of target gene regions, a variety of taxon-specific primers are essential. In the case of Diptera, many primers are used time and time again despite the fact that they were designed originally for use in nondipteran taxa. These primers are often not adequate to amplify and sequence target gene regions in dipteran taxa, leading to considerable frustration and waste. Diptera-specific primers for a variety of gene regions are needed to reduce time and effort spent and to increase sequencing success in molecular phylogenetic research.

Our purpose is to review unique PCR amplification primers that have been developed and published as part of research on Diptera phylogenetics. We have chosen eleven gene regions that include the most commonly sequenced gene regions as well as some that have only recently been developed. These gene regions are small ribosomal subunit (12S), cytochrome *b* (Cytb), cytochrome oxidase *c* subunit I

(COI), 28S ribosomal RNA (28S), alanyl-tRNA synthetase (AATS), the carbamoyl phosphate synthase region of CAD (CAD), elongation factor-1 $\alpha$  (EF-1 $\alpha$ ), 6-phosphogluconate dehydrogenase (PGD), triose phosphate isomerase (TPI), *white*, and *wingless*. To the list of existing primers, we seek to add our own newly designed primers that will provide further options to future researchers that wish to amplify a given gene region for their own dipteran target taxa. We intend to provide sufficient primer alternatives, both old and new, such that any of these eleven gene regions could be amplified and sequenced in any future molecular phylogenetic research involving Diptera.

### Materials and Methods

We surveyed the scientific literature to identify unique PCR amplification primers developed as a part of phylogenetic research involving Diptera. Primers that were different from past primers by at least one nucleotide were included in both primer tables and

Table 2. PCR amplification primers developed to amplify the 12S gene region using Diptera exemplars

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
F14029	ATTTAATAAACSCGTGATACAC	Oliveira et al. (2005)	13897	F (J)	21	<i>Drosophila</i> (Drosophilidae)
S12A	CATTCTAGATACACTTTCCAGT	Han and Ro (2002)	14176	F (J)	22	Tephritoidea
DRMT2279N	GTCATTCTAGATACACTTTCCAGTAC	Jenkins et al. (1996)	14178	F (J)	26	<i>Drosophila</i> (Drosophilidae)
SR-J14197	TACATCTACTATGTTACGACTT	Simon et al. (2006) <sup>a</sup>	14197	F (J)	22	Universal
SR-J-14199	TACATCTACTATGTTACGACTTAT	Kambhampati and Smith (1995)	14199	F (J)	24	Universal
SR-N14220	ATATGTACACATCGCCCGTC	Simon et al. (2006) <sup>a</sup>	14220	R (N)	20	Universal
12Sbi	AAGACGGCAGCGCGGATGTGT	Simon et al. (1994) <sup>a</sup>	14233	F (J)	20	Universal
12Sc	AAGGTGGATTTCGCTAGTAAA	Simon et al. (1994) <sup>a</sup>	14294	R (N)	20	Universal
S12F	CTACACCTTGATCTGATATA	Han and Ro (2002)	14382	F (J)	20	Tephritoidea
DRAT3S	GTAATCCGATAATCCACGATCGACC	Jenkins et al. (1996)	14492	R (N)	24	<i>Drosophila</i> (Drosophilidae)
12Sj	TACAAAACAGGTCCCTCTG	Simon et al. (1994) <sup>a</sup>	14508	F (J)	18	Universal
<b>12S-Dipt-14525R</b>	<b>CGGTATTTTAKTCTDTYACAGAGG</b>	New	<b>14525</b>	<b>R (N)</b>	<b>23</b>	<b>Brachycera</b>
12Se	ACTTAAAAAATTGCGCGGT	Simon et al. (1994) <sup>a</sup>	14540	R (N)	19	Universal
DRAT2S	CTAGGATTAGATACCCTATTA	Jenkins et al. (1996)	14609	R (N)	20	<i>Drosophila</i> (Drosophilidae)
SR-J14610	ATAATAGGGTATCTAATCCTAGT	Simon et al. (2006) <sup>a</sup>	14610	F (J)	23	Universal
12Sai	AAACTAGGATTAGATACCCTATTAT	Simon et al. (1994) <sup>a</sup>	14612	R (N)	25	Universal
12Sair	AAAGGTATCTAATCCTAGTTT	Simon et al. (1994) <sup>a</sup>	14612	F (J)	20	Universal
A12C	CTAGGATTAGATACCCTATTAT	Han and Ro (2002)	14612	R (N)	22	Tephritoidea
SR-N-14594	AAACTAGGATTAGATACCC	Kambhampati and Smith (1995)	14612	R (N)	19	Universal
R14735	AWAAACTAGGATTAGATACCC	Oliveira et al. (2005)	14614	R (N)	21	<i>Drosophila</i> (Drosophilidae)
DRAT1S	AAAAGAAAATTGAATTTATTAGTC	Jenkins et al. (1996)	14696	R (N)	25	<i>Drosophila</i> (Drosophilidae)
SR-N14745	GTGCCAGCAGTCGCGTTATAC	Simon et al. (2006) <sup>a</sup>	14745	R (N)	22	Universal
DRMT1653S	GGTGCCAGCAGTCGCGGTTA	Jenkins et al. (1996)	14768	R (N)	20	<i>Drosophila</i> (Drosophilidae)
<b>12S-Dipt-14771R</b>	<b>GGTGCCAGCAGTYGCCG</b>	New	<b>14771</b>	<b>R (N)</b>	<b>17</b>	<b>Brachycera</b>
12Sh	GACCAAATTGGTGCCAGCAGT	Simon et al. (1994) <sup>a</sup>	14776	R (N)	21	Universal
12Sz	AGTATTGGTAAAATTTGTGCCAGC	Moulton (2000)	14779	R (N)	24	Simuliidae
A12DD	TTTATATGTAATTTTGTGTG	Han and Ro (2002)	14880	R (N)	22	Tephritoidea
12Sgi	AAGTTTTATTTTGGCTTA	Simon et al. (1994) <sup>a</sup>	14939	R (N)	18	Universal
A12X	TTAAAGTTTTATTTGGCTT	Han and Ro (2002)	14942	R (N)	20	Tephritoidea

The 3' location is based on published *D. yakuba* sequence (Clary and Wolstenholme 1985). Direction F, forward; R, reverse (J, majority; N, minority as per Simon et al. 1994). Sequences in bold are newly developed for this study.

<sup>a</sup> Sequences from Simon et al. (1994, 2006) are those matching *Drosophila* without any degeneracy.

gene maps for each gene region. We noted primers that were developed to be species-specific sequencing primers but did not include them in primer tables or maps. In a very few cases, we included primers that were developed as part of non-Diptera research, but that have been used extensively in Diptera molecular phylogenetics.

We also analyzed 1619 DNA sequences obtained for eleven gene regions (Table 1). These sequences were obtained as a part of ongoing phylogenetic research on a number of families of Diptera. The sequence data set included representatives of 40 families of Diptera from across Brachycera (Table 1). Although several other gene regions (e.g., 16S ribosomal DNA, 18S ribosomal DNA, cytochrome oxidase *c* subunit II, and the internal transcribed spacers I and II) have been used in phylogenetic research in the past, they are not included in the current study. We did not have sufficient numbers of sequences from these gene regions in our database to generate alignments and new primer sequences.

All DNA sequences from all fly families available (Table 1) for a given gene region were compiled into a single alignment. Using these alignments, we located small nucleotide sequences that were conserved across the diversity of the sequences included. These candidate primer locations are exact matches, when degenerate sites are included, for all of the taxa in-

cluded in the alignment. We sought to develop primers that are as Brachycera-specific as possible with a minimum of degeneracy. Overall, we also sought to locate potential primers that would, in combination with other new or existing primers, allow amplification and sequencing of each gene region in 500–1,000-bp segments.

Naming of all of the primers we have developed follows a common convention: an abbreviation of the name of the gene region amplified, followed by "Dipt" for Diptera-specific, followed by a location number corresponding to the 3'-most base of the primer compared with a published DNA sequence, followed by F or R for forward or reverse primers, respectively (e.g., 28S-Dipt-3385 F). Although we have not adopted the J and N naming system suggested by Simon et al. (1994), we do include J and N designations in our tables for primers of mitochondrial gene regions. The DNA sequence used to determine the location number within each name varies with the gene region in question.

## Results and Discussion

Existing and newly developed primers for each gene region are summarized in Tables 2–12. In each table, the name as provided in the original reference, sequence, length, direction, and original reference is

**Table 3.** PCR amplification primers developed to amplify the Cytb gene region using Diptera exemplars

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
CB-J-10612	CAAATTAATATTTCAAGATGATGAAA	Simon et al. (1994) <sup>a</sup>	10612	F (J)	26	Universal
CB-J-10612	AAAAAGCTTCCATCAACATCTCAGCATGATGAAA	Gasparich et al. (1995)	10612	F (J)	35	<i>Ceratitis</i> (Tephritidae)
CB-J10621	TCAAGATGATGAAATTTTGGATC	Simon et al. (2006) <sup>a</sup>	10621	F (J)	24	Universal
CB-N10608	CCAACTAATGATCCAAAATTTCA	Simon et al. (2006) <sup>a</sup>	10630	R (N)	23	Universal
CB-N-10671	ACAGGACACATTACATATCG	Gasparich et al. (1995)	10690	R (N)	20	<i>Ceratitis</i> (Tephritidae)
CYT BF2	TACCATCAGGWCAAAATATCATWTTGAG	Lyman et al. (1999)	10916	F (J)	27	Universal
CYT BF1	GGTCAAATATCATTTTGAGKAGCWACWG	Lyman et al. (1999)	10923	F (J)	25	Universal
CYT BF	GGACAAATATCATTTTGAGGAGCAACAG	Lyman et al. (1999)	10923	F (J)	28	Universal
CB-J-10933	TATGTTTTACCTTGAGGACAAATATC	Simon et al. (1994) <sup>a</sup>	10933	F (J)	26	Universal
CB-J10933	GTTTTACCTTGAGGACAAATATC	Simon et al. (2006) <sup>a</sup>	10933	F (J)	23	Universal
CB-N-10920	TCTCAAATGATATTTGTCCTCA	Simon et al. (1994) <sup>a</sup>	10943	R (N)	24	Universal
CB-N-10920	AAACTGCAGCCCTCAGAATGATATTTGTCCTCA	Gasparich et al. (1995)	10943	R (N)	34	<i>Ceratitis</i> (Tephritidae)
CB-N11010	TATCTACAGCAAATCCCTCTCA	Simon et al. (2006) <sup>a</sup>	11010	R (N)	22	Universal
Cytb-9F	ATGAAATTTGAGGGGATTTG	Rao et al. (2006)	11022	F (J)	20	Tipulidae
CytB-Dipt-11035F	GGNTTYKNGTGAAYAAAYCG	New	11035	F (J)	20	Brachycera
CBsunA	AATGTTACAGAAATCA	Dusfour et al. (2004)	11047	F (J)	17	<i>Anopheles</i> (Culicidae)
CytB-Dipt-11074F	CGATTTTYACHHTTYCAITTYATYHTNCC	New	11074	F (J)	29	Brachycera
sunSS	TATCATTCTCAGGAGCC	Dusfour et al. (2007)	11143	R (N)	17	<i>Anopheles</i> (Culicidae)
CytB-Dipt-11146F	GGNTCHAAAYAAAYCCNATNGG	New	11146	F (J)	20	Brachycera
sunE	ATGATTTTACGAATTTGCG	Dusfour et al. (2007)	11230	R (N)	19	<i>Anopheles</i> (Culicidae)
CBsunB	TTAGCTATACATTATCG	Dusfour et al. (2004)	11296	R (N)	17	<i>Anopheles</i> (Culicidae)
Cytb-308R	ACAGGGTTAGCTGGGATAAA	Rao et al. (2006)	11302	R (N)	20	Tipulidae
Forward 11226	GAATGATATTTTTATTGCG	Hodgkinson et al. (2002)	11328	F (J)	20	Psychodidae, Culicidae
CB-N-11328	AGCAAATAAAAAATATCATTC	Simon et al. (1994) <sup>a</sup>	11328	R (N)	21	Universal
CB3-PDR	CAYATTCACCCWGAATGATA	Ready et al. (1997)	11335	F (J)	20	Psychodidae
CB-J11335	CACATTCACCCAGAATGATA	Simon et al. (2006) <sup>a</sup>	11335	F (J)	20	Universal
CB-J-11338	CACATTCACCCAGAATGATATTT	Simon et al. (1994) <sup>a</sup>	11338	F (J)	23	Universal
CB-N-11367	ATAACTCCTCCTAATTTATTAGGAAT	Simon et al. (1994) <sup>a</sup>	11367	R (N)	26	Universal
PDR-WF01	CITTCGTTCTAATTCCTAAT	Hall et al. (2001)	11375	F (J)	18	<i>Chrysomya</i> (Calliphoridae)
CYT BR1	ATTTATTAGCAATWGCCTGAAAAATWG	Lyman et al. (1999)	11379	R (N)	27	Universal
CytB-Dipt-11389R	ACTCCYCCARTTTRITDGG	New	11389	R (N)	20	Brachycera
CYT BR	ATTACTCCTCCTAGCTTATTAGCAATTC	Lyman et al. (1999)	11392	R (N)	28	Universal
CB3-R3A	CGTATTACTCCYCTAECTTRTT	Esseghir et al. (2000)	11395	R (N)	23	Psychodidae
CYT BR2	ATTTGATATACTAAWGCATWACTCCTCC	Lyman et al. (1999)	11411	R (N)	30	Universal
CB-N11526	TTCAACTGGTCAGCTCCAAATCA	Simon et al. (2006) <sup>a</sup>	11526	R (N)	24	Universal
CB-J-11545	ACATCAATTTGAGCTCGACCAGT	Simon et al. (1994) <sup>a</sup>	11545	F (J)	23	Universal
CytB-Dipt-11545R	ACDGGDCGGGCGCCRAITC	New	11545	R (N)	20	Brachycera
PDR-WF03	GCACGACCTGTAGAAGA	Hall et al. (2001)	11551	F (J)	17	<i>Chrysomya</i> (Calliphoridae)
PDR-WR02	GGCTCTTCAACAGCTCG	Hall et al. (2001)	11554	R (N)	17	<i>Chrysomya</i> (Calliphoridae)
CytB-Dipt-11554R	GGRTBTTCADCDGGNCC	New	11554	R (N)	17	Brachycera
PDR-WR04	ATTTACCGCTCATTAACT	Hall et al. (2001)	11675	R (N)	18	<i>Chrysomya</i> (Calliphoridae)
TS1-N-11683	AAATTCATCTTATGTTTTCAAAC	Simon et al. (1994) <sup>a</sup>	11683	R (N)	25	Universal
Reverse 11587	CTTATGTTTTCAAGCATATGCG	Hodgkinson et al. (2002)	11699	R (N)	22	Psychodidae, Culicidae

The 3' location based on published *D. yakuba* sequence (Clary and Wolstenholme 1985). Direction F, forward; r, reverse (J, majority; N, minority as per Simon et al. 1994). Sequences in bold are newly developed for this study.

<sup>a</sup> Sequences from Simon et al. (1994, 2006) are those matching *Drosophila* without any degeneracy.

included for each existing primer. We have also included our newly developed primers, named according to our naming system. We have calculated the location of each primer based on a previously published gene region map. For each primer, existing or newly developed, we have given an approximation of the breadth of taxa for which the primer was developed. In existing primers, this is based on the information given in the original reference and ranges from genus-specific to universal (i.e., primers developed for use in Diptera plus other Insecta). For new primers, the breadth of taxa is determined by the diversity of specimens included in the sequence database used to develop the primers (Table 1). All newly developed primers, except two developed for *wingless*, are developed to be useful across all Brachycera.

**12S.** In their compendium of primers useful in amplifying animal mitochondrial DNA, Simon et al. (1994) included eight unique primers that they had matched with the 12S region of the published *Drosophila yakuba* Burla sequence (Clary and Wolsten-

holme 1985). Four more primers were added in their later animal mitochondrial compendium (Simon et al. 2006). Two modified primers were designed for use across insects and ticks (Kambhampati and Smith 1995). Five unique 12S primers were designed for use in a population dynamics study of *Drosophila* (Drosophilidae) (Jenkins et al. 1996). One additional primer was developed as part of a study of the phylogenetics of Simuliidae (Moulton 2000). Five unique 12S primers were developed in a study on the phylogeny of Tephritoidea (Han and Ro 2005). Oliveira et al. (2005) designed two more primers for use in a study of a *Drosophila* species complex.

We have developed two new 12S primers. The naming of our new primers is based on the published mitochondrial genome of *D. yakuba* (Clary and Wolstenholme 1985). In total, 29 primers are listed and mapped (Fig. 1; Table 2). The entire length of the 12S gene region ( $\approx 800$  bp) can be sequenced using existing primers. The actual length of segments amplified

Table 4. PCR amplification primers developed to amplify the COI gene region using Diptera exemplars

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
UEA1	GAATAATTCGCCATAAATAGATTTACA	Lunt et al. (1996)	1438	F (J)	26	Universal
TY-N-1438d	GAAWAATTCYATAAATWATARATTTACA	Zhang and Hewitt (1996)	1438	F (J)	26	Universal
CCOI-F	GTCTATTGCCCTAAACTTCAGCC	Chen et al. (2004)	1460	F (J)	22	Calliphoridae
TY-J-1460B	TATGCCCTAAACTTCAGCC	Lonsdale et al. (2010)	1460	F (J)	19	Clusiidae
primer 1	TACAATTTATCGCCTAAACTTCAGCC	Sperling et al. (1994)	1460	F (J)	26	Calliphoridae
TY-J-1460	TACAATCTATCGCCTAAACTTCAGCC	Bernasconi et al. (2000b)	1460	F (J)	26	Muscoidea
TY-J-1461	TTTACARTTTACCGCCTATTRTCAGCCA	Winkler et al. (2009)	1461	F (J)	25	Agromyzidae
COI-1	ATCGCCTAAACTTCAGCCAC	Wang et al. (2006)	1462	F (J)	20	<i>Drosophila</i> (Drosophilidae)
LI CDHIM	TCCGCTAAACTTCAGCCATT	Virgilio et al. (2009)	1463	F (J)	20	<i>Dacus</i> (Tephritidae)
COI-F1	CGCCTAAACTTCAGCCACTT	He et al. (2009)	1464	F (J)	20	Drosophilidae
LCOI490-L	GGTCWACWAATCATAAAGATATTGG	Nelson et al. (2007)	1514	F (J)	25	Universal
LCOI490-L	GGTCAACAATCATAAAGATATTGG	Folmer et al. (1994)	1514	F (J)	25	Universal
911	TTTGTAGAAATCATAAAGATATTGG	Guryev et al. (2001)	1514	F (J)	25	Chironomidae
COX1F	TCAACWAATCATAAAGATATTGG	Sayar et al. (2009)	1514	F (J)	23	Tephritidae
LI440d	TYTCAACWAATCATAAAGATATTGG	Van Houdt et al. (2010)	1514	F (J)	25	Tephritidae
Uni Minibar F	TCCACTAATCACAARGATATTGGTAC	Meusnier et al. (2008)	1517	F (J)	26	Universal
CI-J-1535	ATTGGAACCTTATATTTTATATTGG	Scheffer and Wiegmann (2000)	1535	F (J)	26	Agromyzidae
CI-N-1560	TGTTCTACTATTCCGGCTCA	Simon et al. (1994) <sup>a</sup>	1540	R (N)	21	Universal
COI1532	TTYGGAGCTTGATCNGGNATA	Ekrem (2006)	1551	F (J)	21	Tanytarsini (Chironomidae)
CI-J-1632	TGATCAAATTTATAAT	Kambhampati and Smith (1995)	1632	F (J)	16	Universal
Uni Minibar R	GAATATCATAATGAAGCATGAGC	Meusnier et al. (2008)	1668	R (N)	24	Universal
COI-Dipt-1682F	ATTTTTTYATRGTNATRCC	New	1682	F (J)	20	Brachycera
COI-Dipt-1703F	CCHRTHATRATYGGWCGNITYGG	New	1703	F (J)	23	Brachycera
CI-N-1687	CAATTTCCAAATCCTCCAATTAT	Wells and Sperling (1999)	1709	R (N)	23	<i>Chrysomya</i> (Calliphoridae)
CI-J1709	AATGGGGGGTTGGAAATTG	Simon et al. (2006) <sup>a</sup>	1709	F (J)	21	Universal
CI-J-1709	ATAATTTGGAGCATTGGAAATTG	Wells and Sperling (1999)	1709	F (J)	23	<i>Chrysomya</i> (Calliphoridae)
CI-J-1718mod	GGAGGATTTGGAATTTGATTACT	Dallas et al. (2003)	1715	F (J)	23	Universal
CI-J-1718 forward	GGGGGGTTTGGAAATTGATTAGTGCC	Simon et al. (1994) <sup>a</sup>	1718	F (J)	26	Universal
	GGATTTGGAAATTTGATTAGTCTCT	Pradeep Kumar et al. (2007)	1720	F (J)	25	Culicidae
CI-N1738	TTTATTGGTGGGAATGCTATGTC	Simon et al. (2006) <sup>a</sup>	1738	R (N)	23	Universal
COI-Dipt-1751R	GGRAADGCYATRACWGGDMHCC	New	1751	R (N)	23	Brachycera
CI-J-1751 (alias Ron)	GGAGCTCCTGACATGACATTCGCC	Simon et al. (1994) <sup>a</sup>	1751	F (J)	23	Universal
CI-J-1751b	GGATCCCTCGATATAGGYTTTCC	Wells and Sperling (1999)	1751	F (J)	23	<i>Chrysomya</i> (Calliphoridae)
UEA3	TATAGCATTCCCAGCAATAAATAA	Lunt et al. (1996)	1763	F (J)	24	Universal
COI-Dipt-1769F	GCHTTYCCNCGNATRAAAYATRAG	New	1769	F (J)	26	Brachycera
af281	CGAATAAATAAATAAAGATTTTGA	Song et al. (2008)	1776	F (J)	24	Sarcophagidae
L280d	CGAATAAATAAATAAAGATTTTGGAYT	Van Houdt et al. (2010)	1778	F (J)	26	Tephritidae
L280	CGAATAAATAAATAAAGATTTTGGATTA	Van Houdt et al. (2010)	1779	F (J)	27	Tephritidae
Lc/HL-S398F	GTTTACCTCTGCAATTAACCTTA	Chen et al. (2004)	1800	F (J)	23	Calliphoridae
H343	CCAGCTCCGTTTCTACTAT	Van Houdt et al. (2010)	1816	F (J)	20	Tephritidae
CI-N-1843d	GMWARWCGWGRTAWACWGTTCA	Zhang and Hewitt (1996)	1843	R (N)	23	Universal
UEA2	TCAAGATAAAGGAGGATAAACAGTTC	Lunt et al. (1996)	1844	R (N)	26	Universal
K699R	GGGGTAAACTGTGATCC	Wahlberg (2010)	1858	R (N)	19	Nymphalidae
COI-Dipt-1858R	GGRTANACNGYCANCC	New	1858	R (N)	17	Brachycera
CI-J-1859 (alias RonII)	GGTACAGCTTGAACCTGTTTACCCTCC	Simon et al. (1994) <sup>a</sup>	1859	F (J)	26	Universal
CI-N-1958	CGTATATTAATAATTTGTTGAATAA	Scheffer and Wiegmann (2000)	1958	R (N)	25	Agromyzidae
L499	ATTAATATACGATCAACAGGAAT	Van Houdt et al. (2010)	1994	F (J)	23	Tephritidae
H526	ACAATAAAGGTATTCGGTCAAA	Van Houdt et al. (2010)	1999	R (N)	23	Tephritidae
CI-J-2050	ACTCGAATACCTTTATTTGTTTG	Lonsdale et al. (2010)	2024	F (J)	23	Clusiidae
UEA4	AATTTCCGTCAGTTAATAATATAG	Lunt et al. (1996)	2087	R (N)	24	Universal
CI-J-2090	TGTTTTAGCTGGAGCTTACTAT	Bernasconi et al. (2000a)	2090	F (J)	24	Scathophagidae
UEA5	AGTTTTAGCAGGACGAATTAATCTAT	Lunt et al. (1996)	2090	F (J)	24	Universal
COIFg	AGTATTAAGCAGGAGCTTACTAT	Sallum et al. (2002)	2090	F (J)	24	Culicidae
CI-J-2090A	AGTTTTAGCAGGACGAATTAACAT	Bernasconi et al. (2007)	2090	F (J)	24	Dolichopodidae
CI-N-2096d	GANGTATTWARRTTTCGRTCWGTTA	Zhang and Hewitt (1996)	2096	R (N)	25	Universal
CI-J-2101	GGAGCAATTACAATACTATTAACAG	Scheffer and Wiegmann (2000)	2101	F (J)	25	Agromyzidae
COI2121	CCTCCTCCAGCAGGRTCAAAAAAAG	Ekrem (2006)	2121	R (N)	25	Tanytarsini (Chironomidae)
COIF-5'	CCAGCTGGAGGAGGATCC	Palumbi (1996)	2150	F (J)	20	<i>Drosophila</i> (Drosophilidae)
R3 688	CCAAAGAATCAAAATAAATGTTG	Park et al. (2009)	2161	R (N)	23	Calliphoridae
COX1R	CCAAARAATCAAAATAAATGTTG	Sayar et al. (2009)	2161	R (N)	23	Tephritidae
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)	2173	R (N)	26	Universal
HCO2198-L	TAAACTTCAGGGRTGWCCAAARAATCA	Nelson et al. (2007)	2173	R (N)	26	Universal
H2123d	TAWACTTCWGGRTGWCCAAARAATCA	Van Houdt et al. (2010)	2173	R (N)	26	Tephritidae
COI-Dipt-2183F	CARCAYYTATTTTGATTTTGTG	New	2183	F (J)	23	Brachycera
F3 710	CAACATTTATTTTGATTTCTTTGG	Park et al. (2009)	2183	F (J)	23	Calliphoridae
CI-J-2183 (alias Jerry)	CAACATTTATTTTGATTTTGTG	Simon et al. (1994) <sup>a</sup>	2183	F (J)	23	Universal
CI-J-2183C	CAACATTTATTTTGATTTCTTTGG	Bernasconi et al. (2007)	2183	F (J)	23	Dolichopodidae
CI-N-2191mod	CAGGTAAAAATTAATAAATAAATCTCTGG	Dallas et al. (2003)	2188	R (N)	28	Universal
CI-N-2191 (alias Nancy)	CCCCGTAATAAATAAATAAATAAATCTTC	Simon et al. (1994) <sup>a</sup>	2191	R (N)	26	Universal
CI-N-2191	GGTAAAAATTAATAAATAAATCTTC	Kambhampati and Smith (1995)	2191	R (N)	23	Universal
COI-M-2	CCTGATTTCTGACTAATAATATC	Wang et al. (2006)	2191	R (N)	23	<i>Drosophila</i> (Drosophilidae)

Continued on following page

Table 4. Continued

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
cox1R	TTTTTTGGCTCATCCAGAAGT	Castro et al. (2002)	2195	F (J)	20	Universal
CI-J-2195 mod	TCATTTTTGGTCATCCNGAAGT	Lehr et al. (2005)	2195	F (J)	23	Culicidae
CI-J-2195	TTGATTTTTGGTCATCCAGAAGT	Roehrdanz (1993)	2195	F (J)	24	Universal
CI-J2195	TGATTTTTGGTCACCCCTCAAGT	Simon et al. (2006) <sup>a</sup>	2195	F (J)	23	Universal
CI-N-2229	GATTCCTGACTAATAATATGAGAAAT	Lonsdale et al. (2010)	2227	R (N)	26	Clusiidae
CI J2231	TACCTGGATTYGGRAATRAATTC	Lewis et al. (2005)	2231	F (J)	22	<i>Drosophila</i> (Drosophilidae)
Ra DCHIM	TTCCTTTTTCCCGGATTCIT	Virgilio et al. (2009)	2233	R (N)	20	<i>Dacus</i> (Tephritidae)
CI-J-2228	ATTTCTCATATTATTAGTCAAGAATC	Lonsdale et al. (2010)	2252	F (J)	26	Clusiidae
CI-N-2293a	AGTAAACCAATTGCTAGTATAGC	Wells and Sperling (1999)	2293	R (N)	23	<i>Chrysomya</i> (Calliphoridae)
CI-N-2293b	ATGGCATAAATTATTCCTAAAGC	Wells and Sperling (1999)	2293	R (N)	23	<i>Chrysomya</i> (Calliphoridae)
CI-J-2319	TAGCTATTGGAYTATTAGG	Wells and Sperling (2001)	2318	F (J)	19	Calliphoridae
Fly5IP	GGATTATTAGGATTTATTGT	Sallum et al. (2002)	2327	F (J)	20	Culicidae
CI-N-2329 (alias K525)	ACTGTAAATATATGATGAGCTCA	Simon et al. (1994) <sup>a</sup>	2329	R (N)	23	Universal
COIF2 g	GGATTTATTTGGTGGAGCTCA	Sallum et al. (2002)	2336	F (J)	20	Culicidae
sf856	ACTGTAAATATATGATGATCTCA	Song et al. (2008)	2351	F (J)	24	Sarcophagidae
CI N2353	GCTCGTGTATCAACGCTATWCC	Lewis et al. (2005)	2353	R (N)	23	<i>Drosophila</i> (Drosophilidae)
CI-N2353	GCTCGTGTATCAACGCTATWCC	Simon et al. (2006) <sup>a</sup>	2353	R (N)	23	Universal
UEA7	TACAGTTGGAATAGACGTTGATAC	Lunt et al. (1996)	2369	F (J)	24	Universal
COIR2AS	GAAGTAAATAAGCTCG	Sallum et al. (2002)	2371	R (N)	17	Culicidae
P1	CCTGCCTATTTCACTTCAGC	Shi et al. (2005)	2390	F (J)	20	<i>Bactrocera</i> (Tephritidae)
CI-N-2393	CCTGTAGAACGAGCAATAATTATTG	Scheffer and Wiegmann (2000)	2393	R (N)	25	Agromyzidae
UEA-6 mod	TTAATTCCTGTAGGNCAGCAATAATTAT	Lehr et al. (2005)	2395	R (N)	29	Culicidae
UEA6	TTAATWCCWGTWGGNACNGCAATRAATTAT	Lunt et al. (1996)	2395	R (N)	29	Universal
reverse	AAAAATTTTAAATTCAGTTGGAACAGC	Pradeep Kumar et al. (2007)	2404	R (N)	27	Culicidae
COI-Dipt-2411F	GCHACWATAAATATTGCHGTNCC	New	2411	F (J)	23	Brachycera
CI-N-2413	TCARCTRAAAATTTAATTCCTGT	Winkler et al. (2009)	2413	R (N)	24	Agromyzidae
COI-Dipt-2441R	GCHADFDACDTRAAAATTTTRATNCC	New	2441	R (N)	26	Brachycera
CI-J-2441 mod	CCTACAGGAATTAATAATTTTATGATTAGC	Scheffer et al. (2004)	2441	F (J)	32	Agromyzidae
CI-J-2441 (alias Dick) primer 2	CCTACAGGAATTAATAATTTTATGATTAGC	Simon et al. (1994) <sup>a</sup>	2441	F (J)	32	Universal
Brian F	CAGCTACTTTATGAGCTTTAGG	Sperling et al. (1994)	2495	F (J)	22	Calliphoridae
CI-N-2508	CTTCATTTATGAGCAATTAGG	Wahlberg (2010)	2495	F (J)	22	Nymphalidae
CI-N-2514	CTCCAGTTAATCTCCCAACTGTAAT	Scheffer et al. (2004)	2508	R (N)	26	Agromyzidae
	AACTCCAGTTAATCTCCCTAC	Wells and Sperling (2001)	2515	R (N)	21	Calliphoridae
COIF2AS	GCTCATTTCATTATGT	Sallum et al. (2002)	2606	F (J)	17	Culicidae
cox1F	ATTGCAAATCTGCACCTAT	Castro et al. (2002)	2614	R (N)	20	Universal
CI-N-2629	AAATCCTGCTATAATAGCAAATAC	Lonsdale et al. (2010)	2623	R (N)	24	Clusiidae
CI-J-2630	TTTATCAATAGGAGCAGTATTTGG	Bernasconi et al. (2007)	2630	F (J)	24	Dolichopodidae
CI J2636	ATAGGRGCGWTGATTTGCVYATTAT	Lewis et al. (2005)	2636	F (J)	23	<i>Drosophila</i> (Drosophilidae)
F2640	CGWCTMTTGGCTATTATAGCAGG	Oliveira et al. (2005)	2642	F (J)	23	Universal
CI-J-2628	CTATTGGCTATTATAGCAGGATTT	Lonsdale et al. (2010)	2646	F (J)	24	Clusiidae
2672r	CCAGTAAATAATGGGTATCAGTC	Gleason et al. (1997)	2650	R (N)	23	<i>Drosophila</i> (Drosophilidae)
CI-N-2659 (alias Milal)	GTCAAATCCAGTAAATAATGG	Simon et al. (1994) <sup>a</sup>	2659	R (N)	20	Universal
UEA5	AAAAATGTTGAGGAAAAAATGTTA	Lunt et al. (1996)	2735	R (N)	24	Universal
GaRev	AAAAATGCTGGGGAAAGAATGTTA	Otranto et al. (2003)	2735	R (N)	24	Oestridae
UEA 9 mod	GTAATAATTAACATTTTTTCCYCAACA	Bernasconi et al. (2000) <sup>a</sup>	2753	F (J)	26	Muscoidea
UEA9	GTAACCTAACAATTTTTTCCYCAACA	Lunt et al. (1996)	2753	F (J)	26	Universal
GaFor	GTAACATATAACATTTCTCCCCAGCA	Otranto et al. (2003)	2753	F (J)	26	Oestridae
CI-J2756	ACATTTTTCCCCCAACATTT	Simon et al. (2006) <sup>a</sup>	2756	F (J)	20	Universal
UEA9.2	CTAACATTTTTTCTCAACATTTTTTAGG	Sallum et al. (2007)	2762	F (J)	29	Culicidae
COIR2 g	CGTCGAGGTATTCGGCTAA	Sallum et al. (2002)	2764	R (N)	20	Culicidae
CI N2776	TAATCTGAATAAGCTCGNNG	Lewis et al. (2005)	2776	R (N)	20	<i>Drosophila</i> (Drosophilidae)
CI-N2776	GGTAATCTGAATAAGCTCGGAGG	Simon et al. (2006) <sup>a</sup>	2776	R (N)	22	Universal
CI-J-2792b	ATACCTCGGCGACTCTGA	Wells and Sperling (1999)	2792	F (J)	20	<i>Chrysomya</i> (Calliphoridae)
CI-J-2797	CCTCGAGCTTATTCAGATTACC	Simon et al. (1994) <sup>a</sup>	2797	F (J)	22	Universal
R2 1327 primer 3	CAAGTGGCTAAAGCATC	Park et al. (2009)	2800	R (N)	17	Calliphoridae
CO1a-3'	CATTTCAGYGTGTAAAGCATC	Sperling et al. (1994)	2800	R (N)	22	Calliphoridae
Fly10IP	AGTGTAAGCATCAGGTAATC	Palumbi (1996)	2809	R (N)	21	Universal
P2	GCAAATAATGAAATTTGTTCT	Sallum et al. (2002)	2839	R (N)	20	Culicidae
UEA10.2	CAGCTGGAGGGTATTTTGA	Shi et al. (2005)	2952	R (N)	20	<i>Bactrocera</i> (Tephritidae)
TL2-N-3013	TTATTAGTTAATAAYGGTARTTCTG	Sallum et al. (2007)	2984	R (N)	25	Culicidae
	TCCACATATAATCTGCCATATTAG	Wells and Sperling (1999)	3013	R (N)	27	<i>Chrysomya</i> (Calliphoridae)
R3037	TYCATTCACATAATCTGGCATATTAG	Oliveira et al. (2005)	3013	R (N)	26	Universal
TL2-N-3014 mod	AATGCACATAATCTGCCATATTAG	Lehr et al. (2005)	3013	R (N)	23	Culicidae
TL2-N-3014 (Pat)	TCCATTGCACATAATCTGCCATATTAG	Simon et al. (1994) <sup>a</sup>	3014	R (N)	25	Universal
UEA10	TCCAATGCACATAATCTGCCATATTAG	Lunt et al. (1996)	3014	R (N)	25	Universal
TL2-N-3015	ATTGCACATAATCTGCCATATTAG	Lonsdale et al. (2010)	3012	R (N)	24	Clusiidae
TL-N-3017	CTFAAATCCATTCGCAATAATCTGCCATA	Scheffer et al. (2004)	3017	R (N)	28	Agromyzidae
CCOI-R	CCATTGCACATAATCTGCCA	Chen et al. (2004)	3019	R (N)	19	Calliphoridae
COI-2	TCCATTGCACATAATCTGCCA	Wang et al. (2006)	3019	R (N)	20	<i>Drosophila</i> (Drosophilidae)
CULR	TGAAGCTTAAATTCATTGCACTAATC	Dyer et al. (2008)	3024	R (N)	26	Glossinidae

The 3' location based on published *D. yakuba* sequence (Clary and Wolstenholme 1985). Direction F, forward; R, reverse (J, majority; N, minority as per Simon et al. 1994). Sequences in bold are newly developed for this study.

<sup>a</sup> Sequences from Simon et al. (1994, 2006) are those matching *Drosophila* without any degeneracy.

Table 5. PCR amplification primers developed to amplify the 28S gene region using Diptera exemplars

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
re28AB	ACTACCCCTGAATTTAAGCA	Bertone et al. (2008)	3334	F	21	Diptera
D1F	CCSCGTAAYTTAAGCATAT	Friedrich and Tautz (1997)	3337	F	20	Diptera
re28A	AGCGGAGGAAAAAGAAAC **	Bertone et al. (2008)	3359	F	17	Diptera
D1 SP	GGGAGGAAAAGAAACTAAC	Moulton (2000)	3363	F	19	Simuliidae
28y	CAAGGATTCCTCTAGTAGCC	Stireman (2002)	3382	F	20	Tachinidae
28S-Dipt-3385F	GGATTTTCTTAGTAGCGGCC	New	3385	F	20	Diptera
28y	CTAACAAAGGATTTTCTTAGTAGCGGCCGAGT	Hillis and Dixon (1991)	3388	F	30	Universal
28y	CTAACAAAGGATTTTCTTAGTAGCGGCCGAGC	Tachi and Shima (2010)	3388	F	30	Tachinidae
28S-Dipt-3394F	GTAGCGCGGAGCGAAAAAG	New	3394	F	18	Diptera
CP12	TGGATCCAGTCGCTGTTCCTGATAGTGCAG	Porter and Collins (1996)	3570	F	31	Anopheles (Culicidae)
28kk	ACTAGGATTAACCTAAGTACCG	Hillis and Dixon (1991)	3638	F	21	Universal
28kk-1	ACCGATAGTAAACCAAGTACCG	Tachi and Shima (2010)	3638	F	21	Tachinidae
28S-Dipt-3661F	GGGAAAGTTGAAAAAGAACTC	New	3661	F	20	Diptera
D1R	ACTCTCTATTACRAGTTCCTTS	Friedrich and Tautz (1997)	3673	R	22	Diptera
28Sforward	ACAGAGACTTCAAGACTACCTG	Castro et al. (2002)	3686	F	22	Universal
D2a	ACGTGAAACTGCTTAGAGGTT	Ruiz Linares et al. (1991)	3702	F	21	Drosophila (Drosophilidae)
re28B	CCCGTCTTGA AACCGGACC <sup>o</sup>	Bertone et al. (2008)	4067	F	20	Diptera
28Sreverse	TTGCTCCGTGTTTCAAGACGGG	Castro et al. (2002)	4069	R	22	Universal
28z	AGACTCCTTGGTCCGCTTTTCAAGAC	Hillis and Dixon (1991)	4076	R	26	Universal
D3 PCR	TTGAAACACCGGACCAAGGAGCTCA	Moulton (2000)	4077	F	24	Simuliidae
CP15	GTGAATCTTGGTCCGCTTTTCAAGACGGG	Porter and Collins (1996)	4077	R	30	Anopheles (Culicidae)
D2b	ATGTTAGACTCCTTGTGCCGT	Ruiz Linares et al. (1991)	4081	R	21	Drosophila (Drosophilidae)
D3 SP	GCATAGTTCACCATCTTTC	Moulton (2000)	4285	R	19	Simuliidae
10i	CTGCAATCGATTGTCTAGA	Pawlowski et al. (1996)	4358	F	19	Culicomorpha
28b	TCGGAAAGGAAACCGACTCA	Stireman (2002)	4413	R	20	Tachinidae
0ic	GAAGTTTCCTCAGGATAGC	Pawlowski et al. (1996)	4431	F	20	Culicomorpha
re28C	CCGAAGTTCCCTCAGGATAGC <sup>o</sup>	Wiegmann et al. (2000)	4431	F	22	Diptera
28S-Dipt-4534F	CCTATTCTCAAACCTTTAAATGGG	New	4534	F	23	Diptera
28S-Dipt-4610F	GGGCCACTTTTGGTAAAGCAG	New	4610	F	20	Diptera
12r	CCAGTTCCTGCTTACCAA	Pawlowski et al. (1996)	4616	R	17	Culicomorpha
12i	GTAAGCAGACTGGTGTCT	Pawlowski et al. (1996)	4620	F	18	Culicomorpha
28S-Dipt-4632R	GGTTCATCCCAACGCGCC	New	4632	R	18	Diptera
S28E	AGCAGACCGTGGACATCGGA	Han et al. (2002)	4721	F	20	Tephritoidea
11	GTTACRCACTCCTTARCRG <sup>o</sup>	Pawlowski et al. (1996)	4749	R	19	Culicomorpha
D6 SP	CGCTAAGGAGTGTGTAAC	Moulton (2000)	4749	F	18	Simuliidae
28ee	ATCCGCTAAGCAGTGTGTAACAACTCACC	Hillis and Dixon (1991)	4757	F	29	Universal
A28D	ACITTAAGCCCATCCATTIT	Han et al. (2002)	4797	R	20	Tephritoidea
re28P	TGGTATGCGTAGAAGTGTGTC	Wiegmann et al. (2000)	4906	F	23	Diptera
28P	GGCTTAGCCAAAGCACTTCTAGGC	Wiegmann et al. (2000)	4913	R	24	Diptera
28S-Dipt-4964F	GGTGGTAGTACGCAAAATATCC	New	4964	F	21	Diptera
14i	GGATGRCCTAAGTGGGA	Pawlowski et al. (1996)	4992	F	16	Culicomorpha
28S-Dipt-4997F	GGAGGACTGAAAGTGGAGAAGG	New	4997	F	21	Diptera
D7F	CTGAAGTGGAGAAGCGGT	Friedrich and Tautz (1997)	4999	F	17	Diptera
S28G	GAAGTGGAGAAGGTTTCGT	Han et al. (2002)	5004	F	20	Tephritoidea
D7int1	AGGGTTTCGTGTGAACAG	Friedrich and Tautz (1997)	5012	F	18	Diptera
A28F	TGGAACCGTATTCCCTTTCCG	Han et al. (2002)	5150	R	20	Tephritoidea
28S-Dipt-5161F	CGGTTCCAATTCCGTAACC	New	5161	F	19	Diptera
D7int2	TTCCAAACCMATATCTC	Friedrich and Tautz (1997)	5181	R	16	Diptera
D7int3	CGATTTTCAAGGCTCC	Friedrich and Tautz (1997)	5378	R	15	Diptera
re28D	CCGAGCTGCTCTCCAAG	Wiegmann et al. (2000)	5438	F	18	Diptera
15i	TCTATCGACTAGAGACTC	Pawlowski et al. (1996)	5461	R	18	Culicomorpha
A28HL	CTTACCTACATTATTCTATCGACT	Han et al. (2002)	5475	R	24	Tephritoidea
D7R	GACTTCCTTACCTACAT	Friedrich and Tautz (1997)	5482	R	18	Diptera
28S-Dipt-5497F	GGAAGTCGGCAAATTAGATCCG	New	5497	F	22	Diptera
28E	CCTTATCCCGAAGTTIACG	Wiegmann et al. (2000)	5513	R	18	Diptera
28II	GATCCGTAACCTCGGGATAAGGATTGGCTC	Hillis and Dixon (1991)	5521	F	30	Universal
28S-Dipt-5532R	CTCAATCTTCAGAGCCAATCC	New	5532	R	21	Diptera
D8 SP	GCACCTGGGAGAAATCA	Moulton (2000)	5842	R	17	Simuliidae
28F	CAGAGCACTGGGAGAAATCAC	Lonsdale et al. (2010)	5846	R	22	Diptera
28v	AAGGTAGCCAAATGCCTCATC	Hillis and Dixon (1991)	5930	F	21	Universal
D9 SP	AGCCAAAATGCCCTGTATC	Moulton (2000)	5933	F	18	Simuliidae
re28H	CTACTATCCAGCCAAACC <sup>o</sup>	Wiegmann et al. (2000)	6000	F	18	Diptera
28S-Dipt-6018R	GCCCGTTCCTTTGGCTGTGG	New	6018	R	20	Diptera
28I	GGGTCTTCTTTCCCGCT	Lonsdale et al. (2010)	6047	R	18	Diptera
21	GTCARCTCAAMAGCGTC	Pawlowski et al. (1996)	6060	R	18	Culicomorpha
D8 PCR	TTAGACTCAAGTCAAAGGGTCTTCT	Moulton (2000)	6065	R	27	Simuliidae
28w	CCTTTGAGCTTACTCTAATCTG	Hillis and Dixon (1991)	6069	F	24	Universal
D10e	TCAAATACCATCTCT	Ruiz Linares et al. (1991)	6154	F	17	Drosophila (Drosophilidae)
re28Q	GGACATTGCCAGGTAGGGAGTT	Wiegmann et al. (2000)	6406	F	22	Diptera
28Q	AACTCCCTACTCGCAAT	Yang et al. (2000)	6406	R	18	Diptera
D10d	CCGCCCACTCAAACCTCC	Ruiz Linares et al. (1991)	6418	R	19	Drosophila (Drosophilidae)
D10 SP	TACCGCCCCAGTCAAAC	Moulton (2000)	6420	R	17	Simuliidae
D10 PCR	TGAGAGATGTAACCGCCCCAGTCAA	Moulton (2000)	6429	R	24	Simuliidae
28S-Dipt-6462F	GGTGTCCCAAGGCCAGCTCAG	New	6462	F	21	Diptera
28S-Dipt-6565F	CGGCCTATCCATCCTTTTGG	New	6565	F	20	Diptera

Continued on following page

Table 5. Continued

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
28J	CCCTGTGGTAACITTTCT	Lonsdale et al. (2010)	6610	R	18	Diptera
28S-Dipt-6635F	GGCTTGTGGCGCCAAAGCC	New	<b>6635</b>	F	19	Diptera
28S-Dipt-6647R	CGTCGCTATGACCGTTGGCC	New	<b>6647</b>	R	21	Diptera
28K	GAAGAGCCGACATCGAAG <sup>a</sup>	Wiegmann et al. (2000)	6678	R	18	Diptera
28x	GTGAATTTGCTTCATCAATGTAGGAAGAGCC	Hillis and Dixon (1991)	6702	R	32	Universal
28S-Dipt-6723F	CCAAGCGTTGGATTGTTACCC	New	<b>6723</b>	F	22	Diptera
28M	AACCCAGCTCACGTTCCC	Lonsdale et al. (2010)	6747	R	18	Diptera
28j	AGTAGGGTAAAACCTAACCT	Hillis and Dixon (1991)	6782	R	19	Universal
28S-Dipt-6834F	GCGTAGTACGAGAGGAACCC	New	<b>6834</b>	F	20	Diptera
28S-Dipt-6916R	GAGCGCTTCAGGCATAATCC	New	<b>6916</b>	R	20	Diptera
re28X	CGCCTCTAAGTCCGATATCCC <sup>a</sup>	Wiegmann et al. (2000)	6930	F	20	Diptera
28S-Dipt-7176R	CCACTTACAACACCTTGCC	New	<b>7176</b>	R	19	Diptera
28Zc	TGGATCGCAGTATGGCAGCT	Bertone et al. (2008)	7202	R	20	Diptera
28Z	GCAAAGATAAGCTTCAGTGC	Wiegmann et al. (2000)	7220	R	21	Diptera

The 3' location based on published *D. melanogaster* sequence (Tautz et al. 1988). Direction F, forward; R, reverse. Sequences in bold are newly developed for this study.

<sup>a</sup> Reverse-complement versions of these primers have been published with the same location and opposite direction.

and sequenced will vary across taxa due to expansion segments within the gene region.

**Cytb.** Simon et al. (1994) presented eight primers that could be used to amplify the Cytb gene region across Animalia. Their later update (Simon et al. 2006) added six more primers. Gasparich et al. (1995) designed three unique Cytb primers as part of a project to sequence the mitochondrial DNA of *Ceratitidis capitata* (Wiedemann) (Tephritidae). A phylogeographic study of Psychodidae (Ready et al. 1997) included one unique Cytb primer. Although working on triatomine bugs (Hemiptera: Reduviidae), Lyman et al. (1999) designed six primers for the Cytb region based on GenBank sequences of three Diptera species plus the sequences of a bee, a locust, and a crustacean. In another molecular phylogenetic study of Psychodidae, Essegir et al. (2000) presented a new primer. While developing molecular markers for Old World populations of *Chrysomya* (Calliphoridae), Hall et al. (2001) developed four unique primers. Two more Cytb primers were developed as part of population genetics studies of Psychodidae and Culicidae (Hodgkinson et al. 2002). Analyses of the population genetics of *Anopheles* (*Cellia*) included four unique primers

specific to Culicidae as well as several primers specific to individual Cytb haplotypes (Dusfour et al. 2004, Dusfour et al. 2007). A study of invasive species of Tipulidae included two unique Cytb primers (Rao et al. 2006).

We have developed five new primers for the Cytb gene region. The naming is based on the published mitochondrial genome of *D. yakuba* (Clary and Wolstenholme 1985). In total, 42 primers are listed and mapped (Fig. 2; Table 3). The entire length of the gene region ( $\approx 1035$  bp) can be sequenced using the listed primers. There are no known introns within the Cytb gene region.

**COI.** A COI primer designed using Diptera sequence data were first developed by Roehrdanz (1993). Folmer et al. (1994) designed what are now considered the "barcoding region primers," perhaps the two most commonly used COI primers for use across Metazoa. They were developed using *Drosophila* (Drosophilidae) and *Anopheles* (Culicidae) sequences among others. Research on forensically important species of Calliphoridae (Sperling et al. 1994; Wells and Sperling 1999, 2001; Chen et al. 2004; Nelson et al. 2007; Park et al. 2009) has introduced nineteen

Table 6. PCR amplification primers developed to amplify the AATS gene region using Diptera exemplars

Primer Name	Sequence	First reference	3' location	Direction	bp	Developed for
AATS-Dipt-463F	ATGAAHCARTTYAARCC	New	<b>463</b>	F	17	Brachycera
1F40	GNATGAAYCARTTYAARCCNAT	Feng-Yi Su et al. (2008)	466	F	22	Universal
unnamed	CGATCCCAACAGCCGARATGTCCAA	Feng-Yi Su et al. (2008)	505	F	24	Universal
AATS-Dipt-547F	CARAARTGYATHCCGNGCHGG	New	<b>547</b>	F	20	Brachycera
AATS-Dipt-559F	CGNGCHGGHGHGAARCAAYAA	New	<b>559</b>	F	20	Brachycera
AATS-Dipt-598F	GGNAARCAYGINTAYCAYAC	New	<b>598</b>	F	23	Brachycera
2F	TAYCAYCAYACNTTYTYGARATG	Regier (2008)	611	F	24	Universal
AATS-Dipt-631F	ATGYTNGGHAMYTGGTCNTTYGG	New	<b>631</b>	F	23	Brachycera
AATS-Dipt-828R	CCCATYTCCCARAARTTRTC	New	<b>828</b>	R	20	Brachycera
AATS-Dipt-840R	GGNCCNVTYTCNCCCATYTCCC	New	<b>840</b>	R	22	Brachycera
4F	ATGAARGAYAAYYTYTGGGARATGGG	Regier (2008)	847	F	26	Universal
unnamed	ATGAACACCAGATTCCAGATYTCCA	Feng-Yi Su et al. (2008)	946	R	25	Universal
AATS-Dipt-962R	CGATTTRWAYTCWRTRAANACHARRTTCC	New	<b>962</b>	R	28	Brachycera
1R244	CATNCCRCARTCNATRTGYTT	Feng-Yi Su et al. (2008)	1017	R	21	Universal
5R	GGRAANCCRTANCTRTCRTA	Regier (2008)	1677	R	20	Universal

The 3' location based on published *D. melanogaster* sequence (Adams et al. 2000). Direction F, forward; R, reverse. Sequences in bold are newly developed for this study.

Table 7. PCR amplification primers developed to amplify the CAD gene region using Diptera exemplars

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
CAD-60F	GARGTNGTNTTYCARACNCGNAT	Lonsdale et al. (2010)	142	F	23	Clusiidae
CAD-Dipt-144F	TGTNTTYCARACNCGGATGG	New	144	F	20	Brachycera
54F	GTNGTNTTYCARACNCGGATGGT	Moulton and Wiegmann (2004)	145	F	23	Eremoneura
68F	GGATCGTTCATATCGTGACACA	Barr and Wiegmann (2009)	187	F	21	Ceratitidae (Tephritidae)
CAD-Dipt-757F	AGYAAAYGGNCCGGHGYCC	New	757	F	20	Brachycera
320F	ATHHTYGGNATYTYGTTGGNCAYCA	Moulton and Wiegmann (2004)	844	F	26	Eremoneura
CAD-Dipt-844R	TGRTGDCCYARRCADATNCC	New	844	R	20	Brachycera
287nR	TTRTGNCCNKRTRTRCCRTA	Regier (2008)	844	R	20	Universal
338F	ATGAARTAYGGYAATGCGTGCHCAYAA	Moulton and Wiegmann (2004)	907	F	26	Eremoneura
CAD-Dipt-964F	ATGACNTNCARAAYCAYGG	New	964	F	20	Brachycera
CAD359R	CCATGGTTTTGWGANGTCAT	Barr and Wiegmann (2009)	964	R	20	<i>Ceratitis</i> (Tephritidae)
AG-360AR	CCATGATTTGTGARGTCAT	Scheffer et al. (2007)	964	R	20	Agromyzidae
AG-360BR	CCRTGRTTYTGTGAYGTGATC	Scheffer et al. (2007)	964	R	20	Agromyzidae
364R	TCNACNGCRAANCCRTGRTTYTG	Moulton and Wiegmann (2004)	976	R	23	Eremoneura
CAD-Dipt-1088F	CCNTAYTTYTCDGTNCARTTYCATCC	New	1088	F	26	Brachycera
356F	CARTTYCAYCCNGARCA	Regier (2008)	1094	F	17	Universal
350R	RTGYTCNGGRTGRAAYTG	Regier (2008)	1095	R	18	Universal
405R	GCNGTRTYCNGGRTGRAAYTG	Moulton and Wiegmann (2004)	1100	R	23	Eremoneura
CAD-Dipt-1100R	GMHGTGRTYTCNGGRTGRAAYTG	New	1100	R	23	Brachycera
CAD-410R	GGNCCNGCNGTRTYCNGGRTG	Lonsdale et al. (2010)	1106	R	23	Clusiidae
CAD-Dipt-1294F	GGNCARGCNGGNGARTTYGA	New	1294	F	20	Brachycera
267fin2F	GCNCCNGARTTYGAYTA	Regier (2008)	1297	F	17	Universal
CAD-Dipt-1326F	GGNTCNCARGCNATHAARGC	New	1326	F	20	Brachycera
581F2	GGWGGWCAAACWGCWYTMMAAYTYGCG	Moulton and Wiegmann (2004)	1507	F	26	Eremoneura
496F	CARACNCCNYTNAAYTYGCG	Regier (2008)	1507	F	20	Universal
581F	GANACTGARGAYMGRAAAATMTTYGC	Moulton and Wiegmann (2004)	1610	F	26	Eremoneura
576R	TCNTCYTCRTTRTTNGCRAA	Regier (2008)	1646	R	20	Universal
CAD-Dipt-1756R	GCRAANCCNGANCCNARNCCNCC	New	1756	R	23	Brachycera
613F	TGGAARGARGTNGARTAYGARGT	Regier (2008)	1864	F	23	Universal
606nR	ACNACYTCRTAYTCNACYTCYTCCA	Regier (2008)	1867	R	26	Universal
680R	AANGCRTNCNGNACMACYTCRTAYTC	Moulton and Wiegmann (2004)	1879	R	26	Eremoneura
CAD-Dipt-1911F	TGYATHACNGTNTGYAAYATGG	New	1911	F	22	Brachycera
267fin3R	TTYTCCATRTTRCANAC	Regier (2008)	1915	R	17	Universal
681F	GARTGYAAYRTNCARTAYGC	Regier (2008)	2065	F	20	Universal
CAD-Dipt-2065F	GGNCARTGYAAYATHCARTAYGC	New	2065	F	23	Brachycera
787F	GGDGTNACNGCNTGYTTYGARCC	Moulton and Wiegmann (2004)	2248	F	26	Eremoneura
806F	CTNCTNAARATGCCNMGNTGGCA	Moulton and Wiegmann (2004)	2287	F	23	Eremoneura
850F	RAAYATHGGHAGTTCCBATGA	Winkler et al. (2009)	2334	F	20	Agromyzidae
CAD-Dipt-2341F	TGGHAGYTCNATGAARAGYGT	New	2344	F	21	Brachycera
843R	GCYTTYTGRAANGCYTCYTCRAA	Moulton and Wiegmann (2004)	2393	R	23	Eremoneura
CAD-Dipt-2393R	GCYTTYTCNAAANGCYTCYTC	New	2393	R	20	Brachycera
843R2	TCNACCATWCKNARWGCYTTYTGRAA	Moulton and Wiegmann (2004)	2408	R	26	Eremoneura
CAD-Dipt-2803F	GTNCCNGCNGARTGCCNCC	New	2803	F	20	Brachycera
CAD-Dipt-2927F	TCNTCNGTNGARTTYGATGG	New	2927	F	21	Brachycera
970F	GARTTYGAYTGCTGYGC	Regier (2008)	2932	F	17	Universal
970R	TRTCTARTCNGTGGAHACRGTYTCNGG	Winkler et al. (2009)	3018	R	28	Agromyzidae
1057F	GTNTCNACNGAYTAYGAYATGTC	Moulton and Wiegmann (2004)	3020	F	23	Eremoneura
CAD-Dipt-3127F	TCNATGGGHHGNCNARYTRCC	New	3127	F	20	Brachycera
1098R	TTNGGNAGYTCNCCNCCCAT	Moulton and Wiegmann (2004)	3130	R	20	Eremoneura
CAD-Dipt-3130R	TTNGGNARYTCNCCNCCCAT	New	3130	R	20	Brachycera
1028R	TTRTNGGNARYTCNCCNCCCAT	Regier (2008)	3133	R	23	Universal
CAD-Dipt-3144F	CCNAAAYAYATHGCNATGG	New	3144	F	19	Brachycera
CAD-Dipt-3202F	CCNGARTCNATHGAYAGYGC	New	3202	F	20	Brachycera
1124R	CATNCGNGARAAYTTARAARGATTYTC	Moulton and Wiegmann (2004)	3227	R	27	Eremoneura
CAD-Dipt-3370F	GGNCCNGCHATGAAYGTNGC	New	3370	F	20	Brachycera
1201F	GARGCNAARGARATYGAAYTNGAYGC	Moulton and Wiegmann (2004)	3504	F	26	Eremoneura
CAD1201Fc	GAAGCNAARGAAATYCATGTGGATGC	Lonsdale et al. (2010)	3504	F	26	Clusiidae
CAD-Dipt-3504F	GCNAAARGARATYGAAYTNGAYGC	New	3504	F	23	Brachycera
CAD-Dipt-3682F	CCNTTYAAYATGCARYTNAATYGC	New	3682	F	23	Brachycera
1278R	TCRTTNTTYTTWGCRAATYAAATGTCAT	Moulton and Wiegmann (2004)	3694	R	26	Eremoneura
CAD-Dipt-3925F	GGHGTNGARATGCCNCTCNACHGG	New	3925	F	23	Brachycera
CAD-Dipt-3943F	GGNCARGTNGCNTGYTTYGG	New	3943	F	20	Brachycera
CAD-Dipt-4000R	GGDATYTGRAADCCNCTNGAYATC	New	4000	R	24	Brachycera
1436R	CCRTGYTCNGCARTARAARTC	Moulton and Wiegmann (2004)	4228	R	20	Eremoneura

The 3' location based on published *D. melanogaster* sequence (Freund and Jarry 1987). Direction F, forward; R, reverse. Sequences in bold are newly developed for this study.

unique COI primers. Simon et al. (1994) included eleven COI primers in their compendium of mitochondrial insect primers; their later compendium (Simon et al. 2006) added six more unique primers. Two modified primers were designed for use across insects and ticks (Kambhampati and Smith 1995). Palumbi

(1996) added two COI primers, modified to suit *Drosophila* (Drosophilidae) sequences, in his book chapter on PCR. Ten unique COI primers were tested across Insecta, including six Diptera species, by Lunt et al. (1996). Three COI primers were deemed to be useful across invertebrates, including *Drosophila*

**Table 8.** PCR amplification primers developed to amplify the EF-1a gene region using Diptera exemplars

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
M3	CACATYAACATTGTCGTSATYGG	Cho et al. (1995)	2103	F	23	Universal
30F	CAYATYAAAYATHGTSCTNATHGG	Regier (2008)	2103	F	23	Universal
rc40	GTCCGTSATYGGWCACGTMGATTTCYGG	Yang et al. (2000)	2118	F	26	Therevidae
40.6F	ATYGARAARTTYGARAARGARGC	Regier (2008)	2199	F	23	Universal
EFs175	GGAAATGGGAAAAGGCTCCTTCAAGTAYGCTYGGG	Stireman (2002)	2237	F	35	Tachinidae
40.71F	TCNTTYAARTAYGCNTGGGT	Regier (2008)	2238	F	20	Universal
EF-SE	TGAGCGTCAGCGTGGTATC	Esseghir et al. (2000)	2275	F	19	Psychodidae
M44-1	GCTGAGCGYGARCGTGGTATCAC	Cho et al. (1995)	2277	F	23	Universal
rcEF4	GARGGTGGTATYACMATTGA	Yang et al. (2000)	2283	F	20	Therevidae
2284-2302(S)	TATYGCCTTTRTGCAAAATTCG	Baker et al. (2001)	2303	F	20	Diopsidae
rcM44.9	CTTGATGAAATCYCTGTGTCC	Cho et al. (1995)	2362	R	21	Universal
42.8R	ATCATRTRTYTDTAARAARTC	Regier (2008)	2370	R	20	Universal
Sam	YGATTGTCCGCCCGGCTACTGCGTGAAT	Moulton (2000)	2429	F	27	Simuliidae
2477-2495(S)	CTTGCTTTTCACHTTGGGTG	Baker et al. (2001)	2495	F	19	Diopsidae
45.71F	GTNCSNGTNAAYAAARATGGA	Regier (2008)	2529	F	20	Universal
<b>EF1a-Dipt-2544F</b>	<b>ATGGAYTCNTCYGARGCCACC</b>	<b>New</b>	<b>2544</b>	<b>F</b>	<b>20</b>	<b>Brachycera</b>
M46-1	GAGGAAATYAARAAGGAAG	Cho et al. (1995)	2582	F	19	Universal
46	TCAGGAAATCAARAAGGAAG	Yang et al. (2000)	2582	F	20	Therevidae
EF46M	GAGGAAATYAARAARGAAGT	Collins and Wiegmann (2002)	2583	F	20	Empidoidea
<b>EF1a-Dipt-2583F</b>	<b>GARCAAATHAARAARGAAGT</b>	<b>New</b>	<b>2583</b>	<b>F</b>	<b>20</b>	<b>Brachycera</b>
<b>EF1a-Dipt-2655F</b>	<b>CCHATYTCYGGHTGGCAYGG</b>	<b>New</b>	<b>2655</b>	<b>F</b>	<b>20</b>	<b>Brachycera</b>
51	CATGTTCTCRTGCCATCC	Yang et al. (2000)	2662	R	18	Therevidae
EF0-5'	TCCGGATGGCACGGCGAGAACATG	Palumbi (1996)	2665	F	24	Universal
rcM51-1	CATRTRTGTCKCCGTGCCAKCC	Cho et al. (1995)	2665	R	21	Universal
Joe-2	CCGTGCTWCAAGGGATGG	Moulton (2000)	2704	F	18	Simuliidae
Joe	CHTGGTWCAGGGATGGAA	Moulton (2000)	2706	F	19	Simuliidae
<b>EF1a-Dipt-2724F</b>	<b>GGYTTYAACCTNAARAACG</b>	<b>New</b>	<b>2724</b>	<b>F</b>	<b>19</b>	<b>Brachycera</b>
M51.9	CARGACGTATACAAAATCGG	Cho et al. (1995)	2832	F	20	Universal
52R	CCDATYTTTRTANACRTCYTG	Regier (2008)	2832	R	20	Universal
EF-1A 5' SP	TGTTTACAAAATTGGCGGTAT	Moulton (2000)	2838	F	21	Simuliidae
Shemp	TCCRATACCNAAARATTTTGTAT	Moulton (2000)	2842	R	21	Simuliidae
EF-1A 3' SP	TTCCAATACCGCCAATTTTG	Moulton (2000)	2843	R	20	Simuliidae
Curly	GTACTGTTCCGATACCGCC	Moulton (2000)	2849	R	19	Simuliidae
rc47	GGAACAGTACCYGTGGGTCC	Yang et al. (2000)	2859	F	20	Therevidae
<b>EF1a-Dipt-2859F</b>	<b>GGHACAGTACHGTNGGTCG</b>	<b>New</b>	<b>2859</b>	<b>F</b>	<b>20</b>	<b>Brachycera</b>
2869-2889(S)	GGTGTDTTGAAACCAGGTTG	Baker et al. (2001)	2889	F	20	Diopsidae
52.4F	TCNGTNGARATGCAYCAG	Regier (2008)	2951	F	19	Universal
52.5R	TCRTGRGTGCATYTCNAC	Regier (2008)	2952	R	17	Universal
2934-2954(A)	CTTCGTGATGCATTTCACCGG	Baker et al. (2001)	2954	R	21	Diopsidae
<b>EF1a-Dipt-2954R</b>	<b>CTTCGTGATGCATTTCACCRG</b>	<b>New</b>	<b>2954</b>	<b>R</b>	<b>21</b>	<b>Brachycera</b>
rcM52.6	CYTCGTCGGTGCATYTCAC	Cho et al. (1995)	2955	R	20	Universal
EF1-5'	GACAACGGTTGGCTTCAACGTGAAGAACC	Palumbi (1996)	3005	F	28	Universal
<b>EF1a-Dipt-3005R</b>	<b>CGTTYTTNACGGTTCAARRC</b>	<b>New</b>	<b>3005</b>	<b>R</b>	<b>19</b>	<b>Brachycera</b>
M52.7	GTCAGGARYTCCGTGGTGG	Cho et al. (1995)	3030	F	20	Universal
EF-SE2	CGGGTGGTTCAGTACCATGA	Esseghir et al. (2000)	3112	R	20	Psychodidae
<b>EF1a-Dipt-3162R</b>	<b>TGRCDDGTGTGRCACATC</b>	<b>New</b>	<b>3162</b>	<b>R</b>	<b>17</b>	<b>Brachycera</b>
EF2-3'	ATGTGAGCGGTGTGGCAATCCAA	Palumbi (1996)	3165	R	23	Universal
EF2	ATGTGAGCAGTGTGGCAATCAA	Stireman (2002)	3165	R	22	Tachinidae
53.5R	ATRTGVGMNGTRTGRCARTC	Regier (2008)	3165	R	20	Universal
rcM53-2	GCAATGTGRGCGTGTGGCA	Cho et al. (1995)	3168	R	20	Universal
EF5	CTCATATCACGTACAGCRAARGG	Yang et al. (2000)	3351	R	23	Therevidae
41.21R	TGYCTCATRTCDGCVACRGCRAA	Regier (2008)	3354	R	23	Universal
rcM4	ACAGCVACKGTYTGYCTCATRTC	Cho et al. (1995)	3366	R	23	Universal

The 3' location based on published *D. melanogaster* sequence (Hovemann et al. 1988). Direction F, forward; R, reverse. Sequences in bold are newly developed for this study.

*melanogaster* Meigen, by Zhang and Hewitt (1997). One unique primer was developed for use in *Drosophila* species phylogenies (Gleason et al. 1997). Nine additional COI primers were developed in studies of the phylogenetics of Agromyzidae and Fergusoninidae (Scheffer and Wiegmann 2000, Scheffer et al. 2004, Winkler et al. 2009). In a pair of papers inves-

tigating the Muscoidea, Bernasconi et al. (2000a,b) introduced unique primers. A study of the systematics of Chironomidae included one COI primer (Guryev et al. 2001). As part of a comparison of molecular evolution between parasitic Diptera and parasitic Hymenoptera, including representatives of 14 dipteran families, Castro et al. (2002) developed two COI primers.

**Table 9.** PCR amplification primers developed to amplify the PGD gene region using Diptera exemplars

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
pgdfor	GGAGCCGACTCGCTNGARGAYATC	Brissou et al. (2004)	1468	F	24	<i>Drosophila</i> (Drosophilidae)
pgdrev	CGCGGCCCTCGTGNCNCCNGGCAT	Brissou et al. (2004)	3151	R	24	<i>Drosophila</i> (Drosophilidae)
PGDF	AARATGGTNCAYAAAYGGNAT	Scott et al. (1993)	3270	F	20	<i>Ceratitis</i> (Tephritidae)
2F	ATHGARTAYGNGAYATGCA	Regier (2008)	3288	F	20	Universal
2.5AF	ATGAARACCCTYGGCATGTC	Winkler et al. (2009)	3333	F	20	Agromyzidae
<b>PGD-Dipt-3495F</b>	<b>GGNACNGGNAARTGGAC</b>	<b>New</b>	<b>3495</b>	<b>F</b>	<b>17</b>	<b>Brachycera</b>
2.5R	ATRC AACNCCRCGCCACAT	Winkler et al. (2009)	3795	R	20	Agromyzidae
<b>PGD-Dipt-3805F</b>	<b>CGNTGYATHATHMGNAGG</b>	<b>New</b>	<b>3805</b>	<b>F</b>	<b>18</b>	<b>Brachycera</b>
PGDR	CTRTNGCNCRAARTARTC	Scott et al. (1993)	4056	R	20	<i>Ceratitis</i> (Tephritidae)
4R	CCNGTCCARTTNGTRTG	Regier (2008)	4107	R	17	Universal

The 3' location based on published *D. melanogaster* sequence (Scott and Lucchesi 1991). Direction F, forward; R, reverse. Sequences in bold are newly developed for this study.

Three analyses of the phylogenetics of Culicidae included eleven unique primers (Sallum et al. 2002, 2007; Pradeep Kumar et al. 2007). Otranto et al. (2003) developed two COI primers in their study of Oestridae. Dallas et al. (2003) developed improved versions of two COI primers as part of their study of Ceratopogonidae. Lehr et al. (2005) included three unique primers in their study of cryptic species of *Anopheles* (Culicidae). Analyses of species of Drosophilidae (Lewis et al. 2005, Oliveira et al. 2005, Wang et al. 2006, He et al. 2009) have included ten unique primers. Two primers were designed based on published gene sequences of several species of *Bactrocera* (Tephritidae) (Shi et al. 2005). Ekrem (2006) designed two primers using sequences of Chironomidae. A phylogenetic study of Dolichopodidae (Bernasconi et al. 2007) included three COI primers. A study of tsetse flies (Glossinidae) yielded one unique COI primer (Dyer et al. 2008). As part of study on universal DNA mini-barcode, Meusnier et al. (2008) developed two new primers. Two more primers were developed as part of research on the systematics of Sarcophagidae (Song et al. 2008). Virgilio et al. (2009) developed two COI primers designed to be specific to the genus *Dacus*

(Tephritidae). Another study of Tephritidae (Sayar et al. 2009) included two unique primers. Also working with Tephritidae, Van Houdt et al. (2010) developed seven unique primers to facilitate DNA barcoding of museum specimens. A phylogenetic study of Clusiidae (Lonsdale et al. 2010) included eight additional COI primers. Wahlberg (2010) and the members of the Nymphalidae Systematics Group published two unique primers for Nymphalidae online that have been successfully used for Diptera.

In addition, many studies have developed species-specific COI primers for use in population genetics and identification studies. We list them here but do not include them in our primer table or map. Species-specific COI primers exist for species in: *Chrysomya*, *Lucilia*, and *Hemipyrellia* (Calliphoridae) (Chen et al. 2004, Saigusa et al. 2005); *Culicoides* (Ceratopogonidae) (Pagès and Sarto i Monteyés 2005, Nolan et al. 2007, Matsumoto et al. 2009, Pagès, Anopheles, Schwenkenbecher et al. 2009); *Aedes*, *Anopheles*, and *Culex* (Culicidae) (Morlais and Severson 2002, Van Bortel et al. 2002, Hemmerter et al. 2007, Pedro and Sallum 2009); *Drosophila* (Drosophilidae) (Spicer 1995, Goto et al. 1999, de Brito et al. 2002, Dyer and

**Table 10.** PCR amplification primers developed to amplify the TPI gene region using Diptera exemplars

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
tabmothF	GTNGGNGGNACYTGAA	Tyshenko and Walker (1997)	2267	F	17	Tabanidae
<b>TPI-Dipt-2276F</b>	<b>GGAAGTGAAGATCAACGG</b>	<b>New</b>	<b>2276</b>	<b>F</b>	<b>19</b>	<b>Brachycera</b>
1F	AAYTGGAARATGAAAYGG	Regier (2008)	2276	F	17	Universal
TPI 111Fb	GGNAAYTGGAARATGAAAYGG	Bertone et al. (2008)	2276	F	20	Diptera
TPI-2	TGGAAGATCAAYGGNGAYAAATGC	Tittiger et al. (1993)	2285	F	23	<i>Culex</i> (Culicidae)
<b>TPI-Dipt-2459F</b>	<b>GGHGCNTTYACNGGNGA</b>	<b>New</b>	<b>2459</b>	<b>F</b>	<b>17</b>	<b>Brachycera</b>
<b>TPI-Dipt-2525F</b>	<b>GGNCAITCNGARCGHCC</b>	<b>New</b>	<b>2525</b>	<b>F</b>	<b>17</b>	<b>Brachycera</b>
<b>TPI-Dipt-2735R</b>	<b>GCCCASCASGGYTCGTASGC</b>	<b>New</b>	<b>2735</b>	<b>R</b>	<b>20</b>	<b>Brachycera</b>
275R	GCCCANACNGGYTCRTANGC	Bertone et al. (2008)	2735	R	20	Diptera
tabR	GCCCANACNGGYTCUTA	Tyshenko and Walker (1997)	2735	R	17	Tabanidae
2R	GCCCANACNGGYTCRTA	Regier (2008)	2735	R	17	Universal
277R	CCDATNGCCCANACNGGYTC	Bertone et al. (2008)	2740	R	20	Diptera
<b>TPI-Dipt-2740R</b>	<b>CCDATYGCCAVMMBGGYTC</b>	<b>New</b>	<b>2740</b>	<b>R</b>	<b>20</b>	<b>Brachycera</b>
mosR	GTCTGGCGTTGACAATCT	Tyshenko and Walker (1997)	3024	R	18	Culicidae
<b>TPIREV</b>	<b>GTCTGGCGTTGACAATCTCG</b>	<b>Tittiger et al. (1993)</b>	<b>3026</b>	<b>R</b>	<b>20</b>	<b><i>Culex</i> (Culicidae)</b>

The 3' location based on published *D. melanogaster* sequence (Shaw-Lee et al. 1991). Direction F, forward; R, reverse. Sequences in bold are newly developed for this study.

**Table 11. PCR amplification primers developed to amplify the *white* gene region using Diptera exemplars**

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
wh-S1	TYGCGNTATGTNCARCARGA	Tachi and Shima (2010)	11275	F	20	Tachinidae
11404S	TYGCGNTATGTNCARCARGAYGA	Baker et al. (2001)	11278	F	23	Diopsidae
white-Dipt-11297F	GAYTNTTYATWGGNTC	New	11297	F	17	Brachycera
white-Dipt-11315F	GGNTCNHTNACNGCNMGNGARCA	New	11315	F	23	Brachycera
white-Dipt-11563R	GGNYTNGAYTCNTTYAYGGC	New	11563	R	20	Brachycera
white-Dipt-11739R	GCNGYNGAYTTYTYDCNTAGT	New	11739	R	22	Brachycera
wh-R	ATGTARTTRTRTGGNCANTGNGCRCC	Tachi and Shima (2010)	11828	R	26	Tachinidae
WZ2E	AAAYTAYAAAYCCNGCNGAYTTYTA	Besansky and Fahey (1997)	11844	F	23	Culicidae
wh-A1	ACYTGNACRTAAAARTCNGC	Tachi and Shima (2010)	11849	R	20	Tachinidae
11975R	ACYTGNACRTAAAARTCNGCNGG	Baker et al. (2001)	11852	R	23	Diopsidae
WZ11X	TTNARRAARAANCCNCCRAA	Besansky and Fahey (1997)	12866	R	20	Culicidae

The 3' location based on published *Drosophila melanogaster* sequence (O'Hare et al. 1984). Direction F, forward; R, reverse. Sequences in bold are newly developed for this study.

Jaenike 2004, Nunes et al. 2008); *Gasterophilus* (Oestridae) (Pawlas-Opiera et al. 2010); *Simulium* and *Prosimulium* (Simuliidae) (Finn et al. 2006, Gaudreau et al. 2010); *Lydella* and *Pseudoperichaeta* (Tachinidae) (Agustí et al. 2005); and *Bactrocera* (Tephritidae) (Yu et al. 2004).

We have developed eight new primers for the COI gene region. The naming is based on the published mitochondrial genome of *D. yakuba* (Clary and Wolstenholme 1985). In total, 141 primers are listed and mapped (Fig. 3; Table 4). The entire length of the gene region ( $\approx 1540$  bp) can be sequenced using listed primers. There are no known introns within the COI gene region.

**28S.** The first published 28S primers specific to Diptera (Hillis and Dixon 1991) were those based on the

published genome of *D. melanogaster* (Tautz et al. 1988). Nine primers were included with little difference in primer sequence across Eukaryote taxa. These primers were also published with reverse-complement primers given separate names. They are not included separately on our map or table. Four primers, designed especially to amplify the D2 and D10 expansion segments of 28S were designed for species of *Drosophila* (Drosophilidae) (Ruiz Linares et al. 1991). Two primers were designed to amplify the D2 expansion segment of species of *Anopheles* (Culicidae) (Porter and Collins 1996). Eight unique primers were designed to amplify the D4–D7 region of 28S for Culicomorpha (Pawłowski et al. 1996). Seven primers were designed to amplify the D1 and D7 expansion segments across Diptera (Friedrich and Tautz 1997).

**Table 12. PCR amplification primers developed to amplify the *wingless* gene region using Diptera exemplars**

Primer name	Sequence	First reference	3' location	Direction	bp	Developed for
1099-1118S	GAAATGCGNCARGARTGYAA	Baker et al. (2001)	1118	F	20	Diopsidae
LepWG1	GARTGYAARTGYCAYGGYATGCTCTGG	Brower and DeSalle (1998)	1136	F	26	Nymphalidae
Wing-Dipt-1145F	GGYATGCTCGNCTCDTGYAC	New	1145	F	20	Brachycera
Foldvari wg1	GTTAGAACATGTTGGATGCC	Földvári et al. (2007)	1166	F	20	Diopsidae
1147-1166S	GTTAGAACWTGYTGGATGCC	Baker et al. (2001)	1166	F	20	Diopsidae
Wing-Dipt-1181F	GGATGCGNYTVGCNAAAYTTYCG	New	1181	F	22	Brachycera
Wing-Dipt-1226F	CGHTTYGAYCGNCGNCTCNGC	New	1226	F	20	Brachycera
B&D wg3	GGATTGCGTGGCGCCACACCGCTCCA	Baker and DeSalle (1997)	1232	F	26	Drosophilidae
Wing-Dipt-1393R	YGATGSCGATCGTATG	New	1393	R	16	Micropezidae
Wg290F	GCWGTTRACTCACAGYATCGC	Pilgrim et al. (2008)	1459	F	20	Vespoidea
1448-1469A	GAATTNCGTGATACACTRTTCC	Baker et al. (2001)	1470	R	22	Diopsidae
1451-1471S	AYAGTGTATCACGNAATTCCG	Baker et al. (2001)	1471	F	21	Diopsidae
Wing-Dipt-1505F	GCACGNGGACCGTARGG	New	1505	F	17	Micropezidae
B&D wg1	CCCCTCGRTACTGAAACGA	Baker and DeSalle (1997)	1539	F	20	Drosophilidae
B&D wg2	GGAGTTCAAGAAGAGTGTCTTTGA	Baker and DeSalle (1997)	1575	R	24	Drosophilidae
1597-1617A	ATTYTTTTTCRAAAARCTTGG	Baker et al. (2001)	1617	R	21	Diopsidae
Wing-Dipt-1703R	CCRCARCACATYARRTRCA	New	1703	R	20	Brachycera
1723-1742A	CGYTCNACNACAATRACCTC	Baker et al. (2001)	1751	R	20	Diopsidae
Foldvari wg2	CGTTCAACGACAATGACCTC	Földvári et al. (2007)	1751	R	20	Diopsidae
Wing-Dipt-1771R	GCAAGCACCACTGGAATGTRC	New	1771	R	21	Brachycera
PompWg2 rev	ACTGCGCAGCACCACTGGAATGTCGA	Pilgrim et al. (2008)	1774	R	26	Vespoidea
LepWG2a	ACTICGCARCACCARTGGAATGTRCA	Brower and DeSalle (1998)	1774	R	26	Nymphalidae
LepWG2	ACTICGCRCACCARTGGAATGTRCA	Brower and DeSalle (1998)	1774	R	25	Nymphalidae
1756-1775A	ACYTRCARCACCARTGRAA	Baker et al. (2001)	1775	R	20	Diopsidae

The 3' location based on published *D. melanogaster* sequence (Rijeswijk et al. 1987). Direction F, forward; R, reverse. Sequences in bold are newly developed for this study.

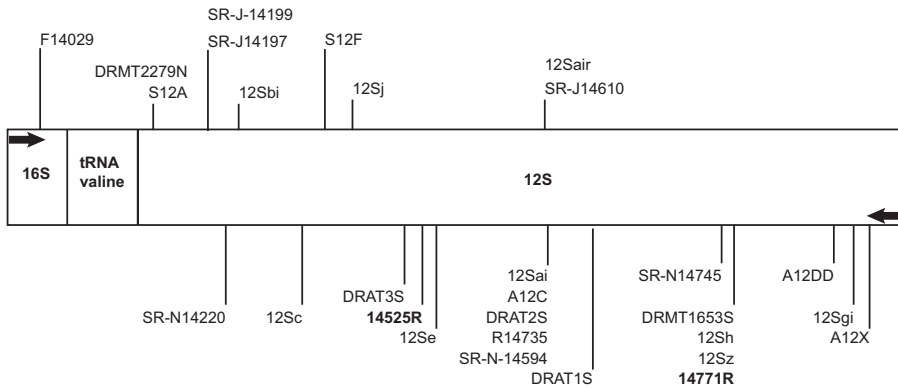


Fig. 1. Map of the 12S gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 2. Primers in bold are newly designed for this study and have had their name abbreviated from “12S-Dipt-xxxxR.” Map is not to scale.

A study of the phylogenetic relationships within Simuliidae yielded nine new 28S primers (Moulton 2000). Four new primers were designed to sequence representatives of Tachinidae (Stireman 2002, Tachi and Shima 2010). Five primers were designed to amplify the D4–D7b region for Tephritoidea (Han et al. 2002). In a comparison of rates of molecular evolution between parasitic Diptera and parasitic Hymenoptera, including representatives of 14 dipteran families, Castro et al. (2002) developed two 28S primers. Nineteen primers, designed for use in Diptera, were developed as a part of a series of studies of the relationships of Therevidae, Tabanomorpha, “lower Diptera,” and Clusiidae (Wiegmann et al. 2000, Yang et al. 2000, Bertone et al. 2008, Lonsdale et al. 2010). Both forward and reverse-complement versions were developed for most of these new primers, and only the unique primer sequences are included in our table and map.

We have developed a further 20 new primers for the 28S gene region. The numbers in the names are based on matching 3’ positions compared with the published ribosomal RNA sequence for *D. melanogaster* (Tautz et al. 1988). In total, 89 primers are listed and mapped

(Fig. 4; Table 5). Although 3’ location numbers of a pair of primers can provide a rough guide to the length of the segment being amplified, variation in expansion segment length, especially D2, D8, and D10, can lead to large fluctuations in amplified sequence length between taxa. These primers can be used in combination to sequence nearly the entire gene, including all expansion segments ( $\approx 3945$  bp for *D. melanogaster*).

AATS. This gene region has only recently been developed for use in dipteran phylogenetics. The compilation of Regier (2008) of nuclear gene region primer sequences for use across Arthropoda includes three AATS primers. Four unique primers are included in a phylogenetic analysis of Sepsidae (Feng-Yi Su et al. 2008). Although none of the primers listed in this paper are attributed to a source, these primers were developed for use in the FLYTREE Assembling the Tree of Life project (Wiegmann et al. 2011). These primers also were used in studies of Asilidae (Dikow 2009) and Schizophora (Gibson et al. 2010b).

We have developed eight new primers for the AATS gene region. The naming is based on the published genome of *D. melanogaster* (Adams et al. 2000). In total, 15

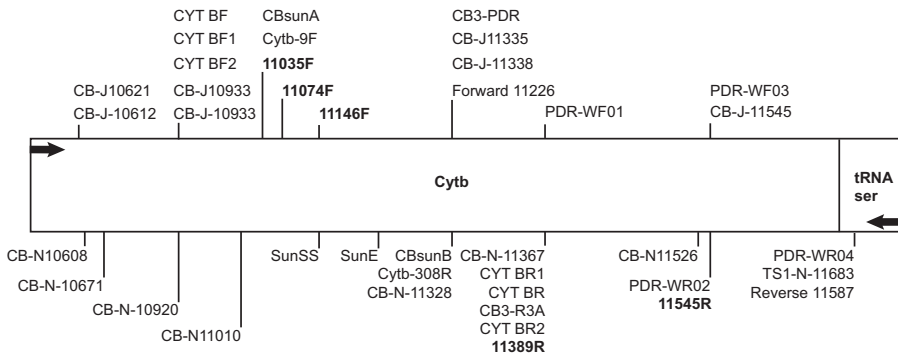


Fig. 2. Map of the Cytb gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 3. Primers in bold are newly designed for this study and have had their name abbreviated from “Cytb-Dipt-xxxxF/R.” Map is not to scale.

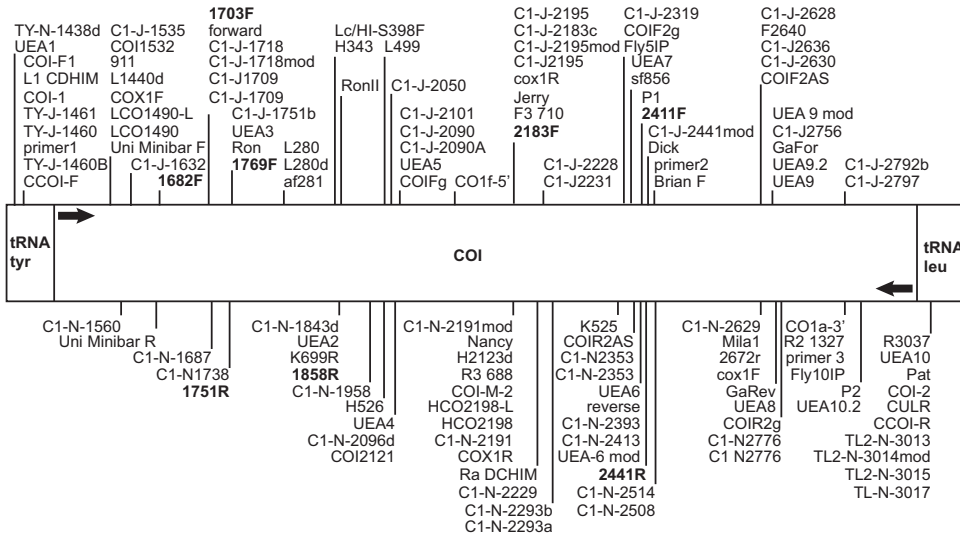


Fig. 3. Map of the COI gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 4. Primers in bold are newly designed for this study and have had their name abbreviated from “COI-Dipt-xxxxF/R.” Map is not to scale.

primers are listed and mapped (Fig. 5; Table 6). A large portion of the gene region ( $\approx 1200$  bp) can be sequenced using listed primers. There are no known introns within the alanyl-tRNA synthetase gene region.

**CAD.** In their analysis of the phylogenetic relationships within Eremoneura, Moulton and Wiegmann (2004) developed eighteen CAD primers and used them to amplify and sequence representatives of 18 families of Diptera. Five more unique primers were added in a pair of studies on the phylogenetics of Agromyzidae (Scheffer et al. 2007, Winkler et al. 2009). Regier (2008) added 14 additional primers, designed to be useful across Arthropoda. Two more

CAD primers were developed as a part of a study involving species of *Ceratitiss* (Tephritidae) (Barr and Wiegmann 2009). Four more CAD primers were developed as a part of research on the systematics of Clusiidae (Lonsdale et al. 2010).

We have developed 25 novel primers using our current data set. Naming of the new primers is based on the published CAD sequence for *D. melanogaster* (Freund and Jarry 1987). In total, 64 primers are listed and mapped (Fig. 6; Table 7). Using the given primers, the entire carbomoylphosphate synthase domain of CAD ( $\approx 4000$  bp for *D. melanogaster*) can be sequenced including a number of taxon-specific introns.

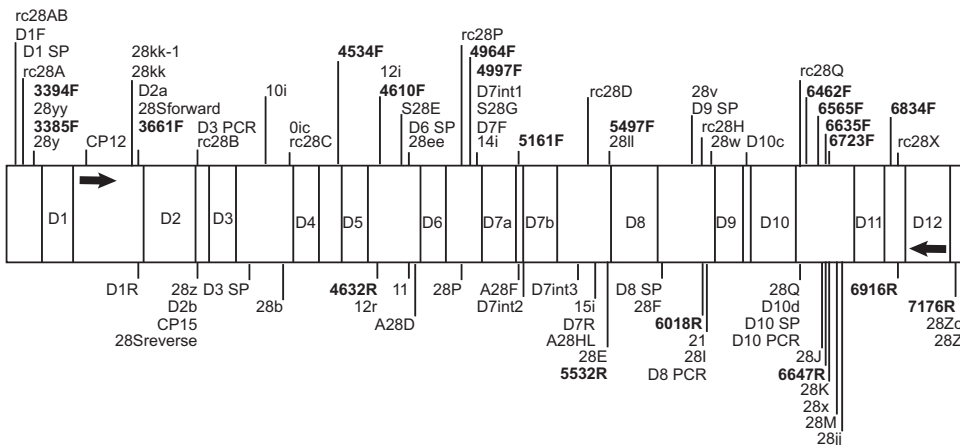
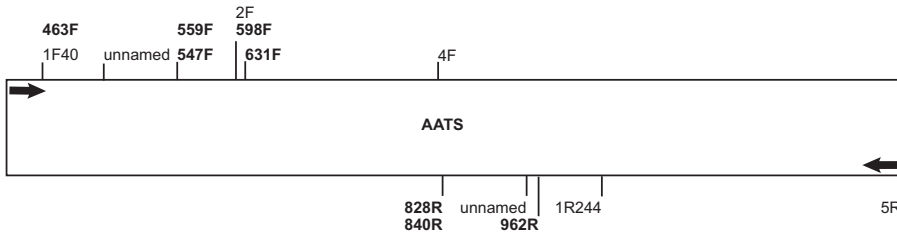


Fig. 4. Map of the 28S gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 5. Primers in bold are newly designed for this study and have had their name abbreviated from “28S-Dipt-xxxxF/R.” Symbols D1–D12 denote expansion segments as identified and labeled in Hancock et al. (1988). Map is not to scale.



**Fig. 5.** Map of the AATS gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 6. Primers in bold are newly designed for this study and have had their name abbreviated from “AATS-Dipt-xxxxF/R.” Map is not to scale.

**EF-1 $\alpha$ .** Although developed as a part of a study on the phylogenetics of heliothine moths, Cho et al. (1995) designed ten EF-1 $\alpha$  primers using published sequences, including *Drosophila*. These primers have been used in many subsequent Diptera phylogenetic papers (Baker et al. 2001, Gibson et al. 2010b). Palumbi (1996) added three unique primers in his book chapter on PCR primers of use across Animalia. A study of the phylogenetic relationships within Simuliidae (Moulton 2000) included seven EF-1 $\alpha$  primers. Research into the systematics of Psychodidae (Essegir et al. 2000) included two unique primers. In their study of higher level phylogenetics of Therevidae, Yang et al. (2000) included six new EF-1 $\alpha$  primers. Four primers were developed as part of a study of the molecular systematics of Diopsidae (Baker et al. 2001). Stireman (2002) developed two unique primers as part of his study of Tachinidae. Collins and Wiegmann (2002) included a modification of an existing primer in their analysis of relationships within Empidoidea. Regier, in the online guide to primer development (Regier 2008), included 10 unique, and quite degenerate, EF-1 $\alpha$  primers, designed to be useful across Arthropoda.

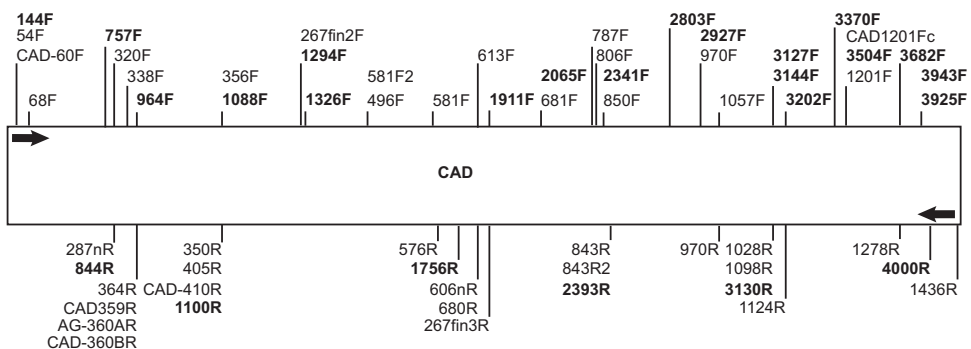
We add eight new EF-1 $\alpha$  primers based on our data set. Naming of the new primers is based on the published EF-1 $\alpha$  sequence for *D. melanogaster* (Hovemann et al. 1988). In total, 53 primers are listed and mapped (Fig. 7; Table 8). Using these primers, almost

the entire coding region of EF-1 $\alpha$  ( $\approx$ 1200 bp) can be amplified and sequenced.

**PGD.** The first PGD primers for Diptera were those developed by Scott et al. (1993) as part of an attempt to map the gene region within *C. capitata* (Tephritidae). Two primers were designed to amplify intron II of PGD in *Drosophila* (Drosophilidae) (Brisson et al. 2004). In his large compendium of primer sequences, Regier (2008) includes two unique primers useful for amplifying PGD across Arthropoda. Two additional primers were developed for a phylogenetic study of Agromyzidae (Winkler et al. 2009).

To these existing primers, we add two new primers. The names are assigned according to the nucleotide numbering system of the PGD gene region of *D. melanogaster* (Scott and Lucchesi 1991). In total, 10 primers are listed and mapped (Fig. 8; Table 9). These primers can be used to amplify most of exon II, all of intron II, and nearly all of exon III of the PGD gene region ( $\approx$ 2600 bp).

**TPI.** The first TPI primers developed for use in Diptera were two developed as part of a study of the gene region within species of *Culex* (Culicidae) (Tittiger et al. 1993). A study of intron development in TPI among representatives of Tabanidae, Culicidae, and *Heliothis* moths included three unique primers (Tyshenko and Walker 1997). A phylogenetic analysis of “lower Diptera” included three more primers (Bertone et al. 2008). The online



**Fig. 6.** Map of the CAD gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 7. Primers in bold are newly designed for this study and have had their name abbreviated from “CAD-Dipt-xxxxF/R.” Map is not to scale.

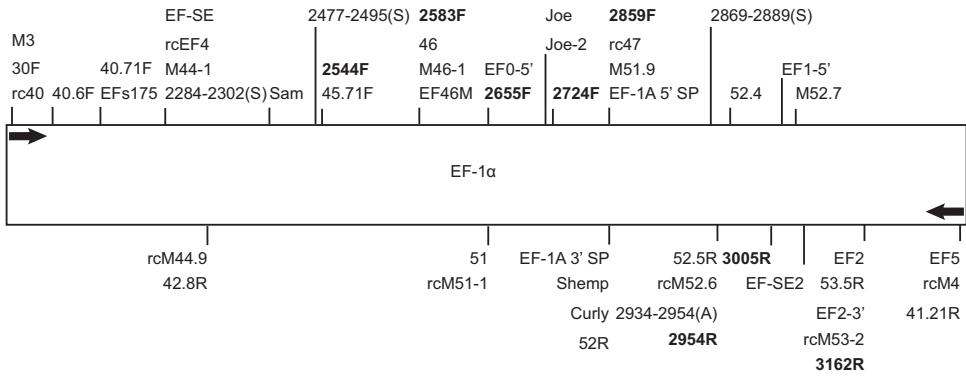


Fig. 7. Map of the EF-1 $\alpha$  gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 8. Primers in bold are newly designed for this study and have had their name abbreviated from "EF1 $\alpha$ -Dipt-xxxxF/R." Map is not to scale.

compendium of primers for nuclear genes includes two TPI primers (Regier 2008). It should be noted that all of the existing primers represent variations on a single forward location and two reverse locations within the TPI gene region.

We have developed five new primers for the TPI gene region, including two new internal sites. The naming is based on the published TPI gene map of *D. melanogaster* (Shaw-Lee et al. 1991). In total, 15 primers are listed and mapped (Fig. 9; Table 10). Using the existing primers, the entire length of the gene region ( $\approx 759$  bp) can be sequenced, including intron locations identified for *Drosophila* (Drosophilidae) (Shaw-Lee et al. 1991) and *Culex* (Culicidae) (Whyard et al. 1994, Tyshenko and Walker 1997).

*White*. A study of relationships among species of Culicidae included two unique *white* primers (Besansky and Fahey 1997). A multigene study of relationships among species of Diopsidae included two additional *white* primers (Baker et al. 2001). In their research on the molecular phylogeny of Tachinidae, Tachi and Shima (2010) added three more primers.

We have developed four new *white* primers. They are named using a modification of the *D. melanogaster* chromosome numbering system (O'Hare et al. 1984).

In the original reference, the gene is numbered from +11050 through 0 to -3050. For ease of use, these numbers have been converted to 1-14100. In total, 11 primers are listed and mapped (Fig. 10; Table 11). Primers exist to amplify nearly all of exon III, and all of intron III, exon IV, intron IV, exon V, and intron V ( $\approx 1500$  bp for *D. melanogaster*). Care should be taken because the presence, size, and location of introns within the *white* gene region vary greatly across Diptera (Gomulski et al. 2001).

*Wingless*. Although the *wingless* gene region has been found to be highly conserved across Brachycera (Mellenthin et al. 2006), it belongs to the *Wnt* family of genes. As many as seven homologs of the *wingless* gene region have been discovered to date in *D. melanogaster* (Sidow 1992, Mellenthin et al. 2006). The homologs seem to be highly conserved such that multiple copies can be amplified using the same primer pairs, leading to an inability to establish homology between DNA sequences of different taxa (Gibson et al. 2010b).

The first *wingless* primers designed specifically for use in Diptera were those developed as part of a study of Hawaiian Drosophilidae (Baker and DeSalle 1997). Seven unique primers were developed specifically for

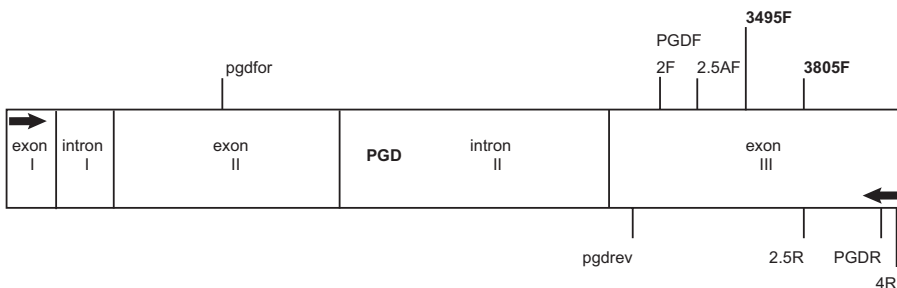
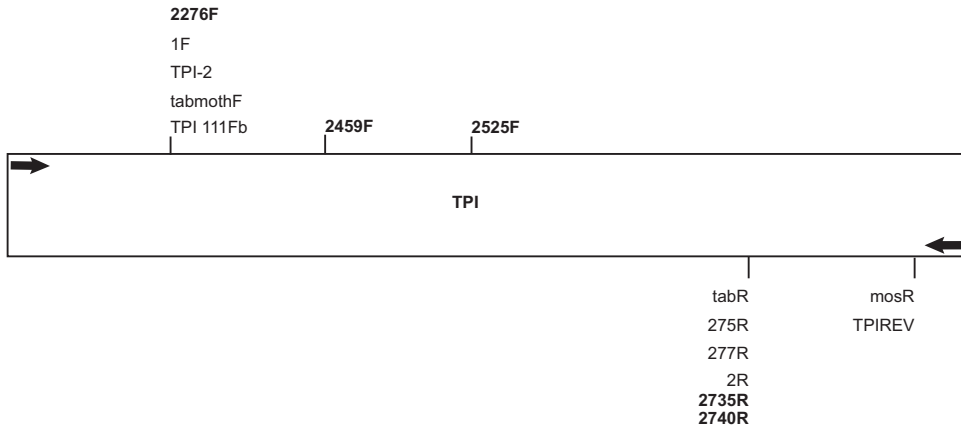


Fig. 8. Map of the PGD gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 9. Primers in bold are newly designed for this study and have had their name abbreviated from "PGD-Dipt-xxxxF/R." Map is not to scale.



**Fig. 9.** Map of the TPI gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 10. Primers in bold are newly designed for this study and have had their name abbreviated from “TPI-Dipt-xxxxF/R.” Map is not to scale.

use in Diopsidae by Baker et al. (2001). Also working within Diopsidae, two additional *wingless* primers were developed by Földvári et al. (2007). Although not designed as a part of phylogenetic studies of Diptera, *wingless* primers designed for use in Nymphalidae (Lepidoptera) (Brower and DeSalle 1998) and Vespoidea (Hymenoptera) (Pilgrim et al. 2008) have been used extensively in dipteran phylogenetic studies (Kotrba and Balke 2006, Kronforst et al. 2007, Gibson et al. 2010b).

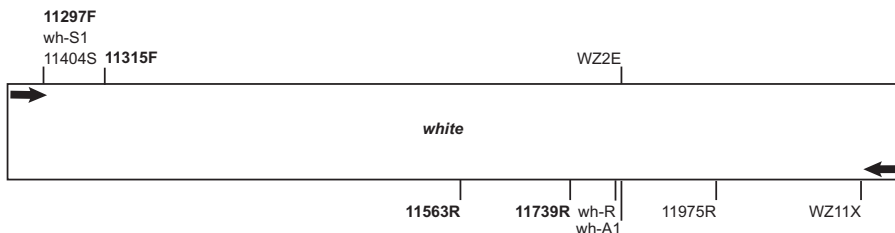
We have developed seven new primers for the *wingless* gene region. Two of these primers were designed to be most useful for specimens of Micropezidae. The naming is based on the published genome of *D. melanogaster* (Rijsewijk et al. 1987). In total, 24 primers are listed and mapped (Fig. 11; Table 12). A large portion of the gene region can be sequenced using these primers (≈650 bp for *D. melanogaster*).

In conclusion, in reviewing past literature, we noticed many instances in which the original references for the primers being used in a study were not easily determined. In some cases, this led to “novel” primers being proposed that had, in fact, been published previously. These instances lead to many problems, not least of which is confusion as to the name of a given

primer. We also noted that in some cases, a primer’s location within the gene region or even the direction of the primer was not included in a publication. These situations lead to difficulty in determining the potential usefulness of a primer in combination with other published primers.

We attempt to improve the present situation with this study. We record 399 previously published unique primers for 11 different gene regions. To this we add 94 primers newly designed as part of this study. We have designed our new primers to be an exact match across all brachyceran families included, yet still with a minimum of degeneracy. Generally, we also have targeted primer locations that will allow all gene regions to be sequenced in as small as 500-bp segments. It should be noted as well that a reverse-complement version of any of our primers could be used as a primer in the same location in the opposite direction. We feel that because our new primers have been designed to match exactly across Brachycera, they should be the first choice for anyone attempting to sequence these gene regions for any brachyceran specimen.

We have attempted to make all existing and new primers as user-friendly as possible through the use of detailed primer tables, gene maps, and a descriptive



**Fig. 10.** Map of the *white* gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 11. Primers in bold are newly designed for this study and have had their name abbreviated from “white-Dipt-xxxxF/R.” Map is not to scale.

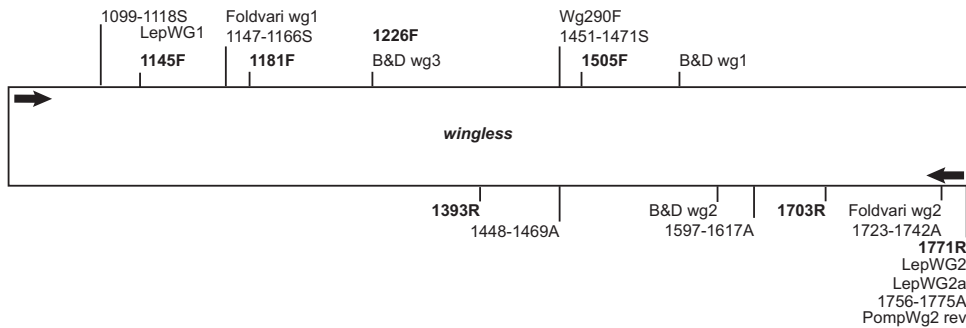


Fig. 11. Map of the *wingless* gene region locating all unique PCR amplification primers designed using Diptera exemplars. Primers above are in the forward direction; primers below are in the reverse direction. Original references and oligonucleotide sequences for each primer are given in Table 12. Primers in bold are newly designed for this study and have had their name abbreviated from "Wing-Dipt-xxxxF/R." Map is not to scale.

naming system for new primers. With this information, anyone attempting to begin or continue a molecular phylogenetic project with dipteran specimens will have a large number of choices of both gene regions and PCR amplification primers. Although our research has been specifically focused on dipteran taxa, we believe that a similar approach could be taken in any other target group of organisms. The cataloguing, mapping, and proper naming of all existing and newly developed PCR amplification primers can only expedite the generation of DNA sequence data for use in phylogenetic study.

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