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Explosive radiation or uninformative genes? Origin and early diversification of tachinid flies (Diptera: Tachinidae)

Isaac S. Winkler [a,b], Jeremy D. Blaschke [c], Daniel J. Davis [a], John O. Stireman III [a,*], James E. O'Hara [d], Pierfilippo Cerretti [e,f], John K. Moulton [c]

[a] Department of Biological Sciences, Wright State University, Dayton, OH 45435, USA
[b] Department of Biology, Linfield College, McMinnville, OR 97128, USA
[c] Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN 37996, USA
[d] Canadian National Collection of Insects, Agriculture and Agri-Food Canada, 960 Carling Avenue, Ottawa, Ontario K1A 0C6, Canada
[e] DAFNAE-Entomology, Università degli Studi di Padova, Viale dell'Università 16, 35020 Legnaro (Padova), Italy
[f] Dipartimento di Biologia e Biotecnologie 'Charles Darwin', 'Sapienza' Università di Roma, Piazzale A. Moro 5, 00185 Rome, Italy

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ABSTRACT

Molecular phylogenetic studies at all taxonomic levels often infer rapid radiation events based on short, poorly resolved internodes. While such rapid episodes of diversification are an important and widespread evolutionary phenomenon, much of this poor phylogenetic resolution may be attributed to the continuing widespread use of "traditional" markers (mitochondrial, ribosomal, and some nuclear protein-coding genes) that are often poorly suited to resolve difficult, higher-level phylogenetic problems. Here we reconstruct phylogenetic relationships among a representative set of taxa of the parasitoid fly family Tachinidae and related outgroups of the superfamily Oestroidea. The Tachinidae are one of the most species rich, yet evolutionarily recent families of Diptera, providing an ideal case study for examining the differential performance of loci in resolving phylogenetic relationships and the benefits of adding more loci to phylogenetic analyses. We assess the phylogenetic utility of nine genes including both traditional genes (e.g., CO1 mtDNA, 28S rDNA) and nuclear protein-coding genes newly developed for phylogenetic analysis. Our phylogenetic findings, based on a limited set of taxa, include: a close relationship between Tachinidae and the calliphorid subfamily Polleninae, monophyly of Tachinidae and the subfamilies Exoristinae and Dexiinae, subfamily groupings of Dexiinae + Phasiinae and Tachininae + Exoristinae, and robust phylogenetic placement of the somewhat enigmatic genera *Strongygaster*, *Euthera*, and *Ceracia*. In contrast to poor resolution and phylogenetic incongruence of "traditional genes," we find that a more selective set of highly informative genes is able to more precisely identify regions of the phylogeny that experienced rapid radiation of lineages, while more accurately depicting their phylogenetic context. Although much expanded taxon sampling is necessary to effectively assess the monophyly of and relationships among major tachinid lineages and their relatives, we show that a small number of well-chosen nuclear protein-coding genes can successfully resolve even difficult phylogenetic problems.

1. Introduction

The observation that resolution of some phylogenetic questions remains problematic despite significant sampling of characters and taxa continues to be a central theme of the phylogenetic literature (Rokas et al., 2005; Wiegmann et al., 2011). This is widely interpreted as a reflection of a pervasive evolutionary pattern: adaptive radiation leading to rapid evolutionary origin of lineages (Rokas et al., 2005; Rokas and Carroll, 2006; Whitfield and Lockhart, 2007; Whitfield and Kjer, 2008). This phenomenon of rapid radiation, characterized by phylogenies with short deep internodes and relatively long terminal branches, has been invoked to explain evolutionary patterns of some of the major lineages of organisms, including eukaryotes (Philippe et al., 2000), metazoans (Rokas et al., 2005), birds (Hackett et al., 2008; McCormack et al., 2013), mammals (Romiguier et al., 2013), angiosperms (Davis et al., 2005), and a great many component lineages within these clades.

However, it has long been recognized that lack of phylogenetic resolution of higher taxa may have multiple and possibly overlapping causes, including simultaneous or rapid speciation events,

* Corresponding author at: Department of Biological Sciences, Wright State University, 3640 Colonel Glenn Hwy, Dayton, OH 45435, USA.
E-mail address: john.stireman@wright.edu (J.O. Stireman III).

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data with insufficient phylogenetic power, and conflicting phylogenetic and non-phylogenetic signal (Rodríguez-Ezpeleta et al., 2007; Walsh et al., 1999). Appropriate sampling of both taxa and genes is important for maximizing phylogenetic signal and for minimizing non-phylogenetic signal in datasets (Cummings and Meyer, 2005). Due to constraints on study design, many current studies inadvertently illustrate the importance of adequately addressing both dimensions of phylogenetic sampling. For instance, some recent studies emphasizing the utility of a taxon-rich design have attempted to reconstruct the phylogeny of major lineages using one or a few easily obtained genes (e.g., Agnarsson and May-Collado, 2008; Bocak et al., 2013; Quicke et al., 2012; Wilson, 2011). In contrast, the burgeoning field of phylogenomics involves intensive sampling of genes, but usually relatively small taxon samples. Experience has shown that study designs involving either extreme frequently show instability between different methods of analyses, failure to recover uncontroversial "test clades," or incongruence with studies employing a more balanced sampling approach (Kumar et al., 2012; Soltis et al., 2004).

Phylogenomic studies are also emphasizing another methodological point long apparent to practitioners of molecular phylogenetics: that markers may differ dramatically in their ability to recover a plausible phylogeny (Salichos and Rokas, 2013). In many phylogenomic studies, exclusion of genes that are demonstrably biased or evolving at a rate inappropriate for the questions being addressed has proven to be important in recovering phylogenetic signal (Jeffroy et al., 2006; Meusemann et al., 2010; Regier et al., 2008). Despite the clear importance of gene choice in inferring robust higher-level phylogenies, this point perhaps remains generally underappreciated among systematists; a large portion of studies still rely on markers of limited utility at the relevant phylogenetic depth. These are often genes encoding ribosomal RNA (i.e., 16S, 18S, 28S) or mitochondrial proteins (i.e., COI, cytb, etc.). Both sets of genes are admittedly useful when applied to appropriate data sets, but mitochondrial genes, especially, are seen as problematic for reconstructing intermediate- to deeper-level phylogenies (Lin and Danforth, 2004). There are practical reasons for these choices, of course, including ease of amplification (especially with poorly-preserved material), availability of comparable sequences from diverse taxa, and published primer sequences. Furthermore, a number of studies have achieved acceptable levels of resolution with multigene ribosomal and mitochondrial data sets (Havird and Santos, 2014; Mallatt et al., 2004; Mallat and Giribet, 2006). Results of mitogenomic studies, many of which also have limited taxon sampling, have shown mixed results (Cameron et al., 2007; Sheffield et al., 2009; Nelson et al., 2012; Zhao et al., 2013), though advocates continue to tout the phylogenetic utility of whole mitochondrial genomes (Wei et al., 2010; Caravas and Friedrich, 2012). To some degree, it is still an open question whether a large number of characters from individually suboptimal genes can effectively resolve difficult phylogenetic problems.

Here we examine the utility of nine genes in reconstructing phylogenetic relationships among a representative set of taxa of the parasitoid fly family Tachinidae and related outgroups (see below). We use both traditional genes (e.g., CO1 mtDNA, 28S rDNA) and genes newly developed for phylogenetic analysis. The family Tachinidae is taxonomically difficult, relatively recent and actively radiating (Cerretti et al., 2014; Crosskey, 1976), providing an ideal case study for examining the performance of different loci in resolving phylogenetic relationships and the benefits (or lack thereof) of adding more loci to phylogenetic analyses. We begin by briefly reviewing the use of nuclear protein-coding genes in insect molecular phylogenetics and discussing the major phylogenetic hypotheses and questions regarding tachinid flies.

1.1. Background

1.1.1. Nuclear protein-coding genes in insect phylogenetics

Primer development and amplification across taxa for nuclear protein-coding markers can be relatively difficult compared to mitochondrial and ribosomal genes. Nonetheless, single-copy nuclear protein-coding genes have historically outperformed ribosomal and mitochondrial protein-coding genes in phylogenetic analyses (Moulton and Wiegmann, 2007; Regier et al., 2008). A number of such markers have been widely used in insect systematics during the last 15 years (e.g., Caterino et al., 2000; Gibson et al., 2011), including EF1-α (Cho et al., 1995), wingless (Brower and DeSalle, 1998), DDC (Fang et al., 1997), PEPCK (Friedlander et al., 1996), histone 3 (Colgan et al., 1998), and LW-opsin (Mardulyn and Cameron, 1999). However, nuclear protein-coding markers may vary significantly in substitution rates and patterns of molecular evolution, and thus in their phylogenetic utility. In fact, the above listed markers tend to show limited variation at the protein sequence level, and thus they derive most of their utility from synonymous mutations. The introduction of CAD (Moulton and Wiegmann, 2004) marked the first time an extensively variable (at the protein level), alignable, single copy, low intron burdened (at least in insects), universal nuclear coding gene became available to the insect molecular systematics community. It has since been widely adopted, with several studies completed within Coleoptera (Jordal and Cognato, 2012; Maddison, 2012; Wild and Maddison, 2008), Diptera (Hash et al., 2013; Petersen et al., 2007, 2010; Reidenbach et al., 2009; Wiegmann et al., 2011), Hymenoptera (Danforth et al., 2006; Duennes et al., 2012; Hedtke et al., 2013; Rightmyer et al., 2013; Sharanowski et al., 2011), Lepidoptera (Gilligan et al., 2013; Kawahara et al., 2009; Regier et al., 2009), Neuroptera (Winterton and Freitas, 2006; Winterton et al., 2010), and the Holometabola (McKenna and Farrell, 2010; Wiegmann et al., 2009).

During the last decade, additional progress was made in finding informative nuclear protein-coding genes for arthropod phylogenetics (e.g., Wild and Maddison, 2008; Gibson et al., 2011), most notably in a large-scale survey culminating in the publications of Regier et al. (2008, 2010). This latter work facilitated the use of several novel genes in Diptera systematics, including PGD, TPI, and AATS (Gibson et al., 2011; Wiegmann et al., 2011). Cho et al. (2011) and Regier et al. (2013) subsequently made full use of the new arthropod markers to reconstruct the phylogeny of the order Lepidoptera. These arthropod and Lepidoptera studies strongly highlighted phylogenetic inconsistency introduced by relying on signal from synonymous substitutions, even the relatively infrequent synonymous first position substitutions (e.g., those coding for Arginine and Leucine; see also Regier and Zwick, 2011).

The advent of high-throughput sequencing and consequent availability of much greater amounts of genomic data is currently driving a phylogenomic revolution in molecular systematics. Though still handicapped to a certain extent by constraints on taxon sampling, phylogenomic studies have already resulted in a rich body of data that can now be exploited for development of new markers by traditional, PCR-based methods. Due to issues with introns, however, amplification of these markers by traditional PCR is not always straightforward or reliable. There is still need for further testing and development of informative, widely amplifiable nuclear protein-coding markers for PCR-based insect phylogenetics. The success of CAD as a phylogenetic marker should encourage this, as should the rapidly growing amount of genomic data available that will facilitate gene choice and primer development.

1.1.2. Tachinid relationships

The parasitic fly family Tachinidae is an ideal test case for a recent rapid radiation representing a difficult phylogenetic problem. Tachinid larvae are internal parasitoids of other insects, including caterpillars, adult and larval beetles, and bugs (Heteroptera), as well as a variety of other insects, and are often agriculturally important enemies of pest insects (Stireman et al., 2006). Adult tachinids show a wide variety of forms, but are often housefly-like in size and appearance. Tachinidae comprise the second largest family of flies, measured by the number of described species (over 8000), but are nested within one of the most recent large radiations of flies, Oestroidea, and probably underwent rapid diversification no earlier than the Oligocene (about 30 million years ago; O'Hara et al., 2013; Wiegmann et al., 2011). The difficulties of tachinid taxonomy and identification are well-known to dipterists and are exacerbated by the recognition of over 1500 currently valid, and often poorly defined, genera (O'Hara, 2012).

The classification of Tachinidae, as well as its separation from related families of Oestroidea, has a convoluted history (O'Hara, 2013), but specialists now largely agree upon a classification with four subfamilies (Tachininae, Exoristinae, Dexiinae, and Phasiinae) and roughly 50 tribes (Cerretti, 2010; Herting, 1984; O'Hara and Wood, 2004) that is supported by data on life history, chaetotaxy, and genitalic morphology (Cerretti et al., 2014; O'Hara, 2013). Despite this considerable progress, monophyly, composition and subfamily affinities of a number of tribes are unresolved, and little is known about tribal relationships within subfamilies. More importantly, evidence supporting each of the subfamilies is weak at best, with only a single putative unambiguous synapomorphy supporting the Dexiinae (Wood, 1987; but see Cerretti et al., 2014) and Phasiinae (Cerretti et al., 2014; Tschorsnig, 1985a), respectively. Two molecular phylogenetic studies have been published on the subfamily Exoristinae (Stireman, 2002; Tachi and Shima, 2010), each providing evidence for monophyly of Exoristinae (limited by outgroup sampling) and certain exoristine tribes. Most recently, a phylogeny of the family using morphological characters (Cerretti et al., 2014) corroborated the general outline of tachinid classification and recovered many recognized groups, though only two subfamilies (Phasiinae and Exoristinae) were monophyletic in most, but not all, analyses. This study also corroborated and quantified the original, persistent problem in tachinid systematics: most characters show high levels of homoplasy, a pattern attributable to an initial rapid radiation of lineages.

A disproportionate part of the uncertainty in tachinid classification and phylogeny centers around the affinities of a few specific tribes, each with only a few genera. Among these are the Strongygastrini (Phasiinae or Tachininae), Eutherini (Phasiinae or Dexiinae), and Acemyini (Exoristinae or Tachininae) (O'Hara, 2013; Cerretti et al., 2014). In each of these cases, conflicting character sets and/or morphological reduction (male phallus) make placement of these groups unclear.

Although the monophyly and composition of Tachinidae are now relatively stable, the question of the origin of tachinids within Oestroidea is not. This question is further complicated by the breakup of the blow fly family Calliphoridae (Rognes, 1997) and the uncertain relationships of its subfamilies that possibly represent distinct lineages, including Rhiniinae, Mesembrinellinae and Bengaliinae (each now often raised to family rank), Polleniinae, and Helicoboscinae. In particular, the identity of the sister group of Tachinidae remains very much uncertain. Phylogenetic studies have varied widely in the proposed sister group of Tachinidae, including Sarcophagidae (Pape, 1992; Rognes, 1997), Calliphoridae (Wiegmann et al., 2011), Mesembrinellinae (Marinho et al., 2012), Polleniinae (Nelson et al., 2012; Singh and Wells, 2013), or a combination of these last two groups (Kutty et al., 2010).

1.2. Study objectives

In this study, we attempt to resolve the basic phylogenetic groupings of Tachinidae with multiple genes in order to answer some of the outstanding questions discussed above about tachinid relationships. At the same time, we wish to test the relative utility of different classes of genes (nuclear ribosomal, mitochondrial protein-coding, and nuclear protein-coding) for phylogenetic reconstruction of taxa that have experienced rapid radiation. Towards these goals, we have developed novel markers that have not been previously used for phylogenetic inference, except possibly as unnamed members of large, genomic datasets, and the performance of these will be especially scrutinized. Specifically, we will ask: (1) Can we resolve relationships within a large, rapid radiation with few exemplar taxa, given enough molecular markers? (2) What is the relative contribution of the different genes and classes of genes to phylogenetic resolution, and how many markers are sufficient? (3) What is the sister group of tachinids, and what are the implications for the evolution of parasitoidism in Oestroidea? (4) What are the relationships between the subfamilies of Tachinidae, and are the subfamilies monophyletic? (5) Where do taxa of uncertain affinity (Acemyiini, Strongygastrini, Eutherini) fit phylogenetically?

2. Methods

To examine the basic relationships among major tachinid lineages and assess the likely sister taxon of the family, we assembled ethanol-preserved material for a set of taxa (Table 1) including ten oestroid taxa representing Calliphoridae (Luciliinae, Melanomyiinae, Mesembrinellinae and Polleniinae), Oestridae, Rhinophoridae, Sarcophagidae and 22 tachinid genera in all four subfamilies, each representing a different tribe. Although there are more than 50 recognized tribes of Tachinidae (Cerretti et al., 2014), the 22 sampled tribes include most major tribes (excluding only three tribes with >100 species), and comprise approximately 80% of tachinid diversity. Genbank sequences for *Musca domestica* were included for outgroup rooting. In some cases, multiple specimens of a genus were extracted, and in a few cases sequences belonging to different species within a genus were concatenated in the data set (noted in Table 1). In these cases, concatenated multigene analyses treated each genus as a single operational taxonomic unit (OTU). Extractions, amplification, and sequencing were carried out in parallel in Stireman Lab at Wright State University and Moulton Lab at University of Tennessee, following different protocols in each lab. Sequences of the previously developed markers 18S, 28S, COI, EF1a (Cho et al., 1995), CAD (Moulton and Wiegmann, 2004), and TPI (triose phosphate isomerase; Tyshenko and Walker, 1997; Bertone et al., 2008) were generated in the Stireman Lab, while three additional genes, discussed below, were sequenced in the Moulton Lab.

2.1. Newly developed genes

Since the introduction of CAD, author JKM has identified and evaluated for obtainability, alignability, and phylogenetic informativeness, several additional genes that are similar to CAD in length and character, but are even more variable and thus powerful. These genes were developed mostly within Diptera, which as a group possesses a rather low intron burden compared to other insects for which nuclear genomes are known. These genes were developed for the express intent of application in phylogenetic studies of Mesozoic- and Cenozoic-aged explosively radiated groups with the hope that their large size and multiple domains experienced nonsynonymous mutations quickly enough to track

Table 1
Taxa sampled and Genbank accession numbers (also see footnotes). Two numbers are given for CAD in cases where the first and second segment were sequenced from different individuals.

Taxon	Subfamily: Tribe (Family: Subfam.)	18S	28S	COI	EF1α	TPI	CAD	LGL	MCS	MAC
Musca domestica L.	Muscidae: Muscinae	DQ133074.1	AJ551427.1	EU815009.1	AF503149.1	GQ265639.1	AY280689.1	KP235350	–	KP722541
Helicobia rapax (Walker)	Sarcophagidae: Sarcophaginae	KP954336	KP954366	KP899680	KP899707	KP954398	KP973918	–	–	–
Macronychia sp.	Sarcophagidae: Miltogramminae	KP954340	KP954370	KP899681	KP899709	KP954402	KP973921	KP235347	KP686291	KP722539
Cuterebra austeni Sabrosky	Oestridae: Cuterebrinae	KP954330	KP954361	JF439549[a]	KP899710	KP954392	KP973914	KP235338	KP686282	KP722532
Cephenemyia jellisoni Townsend	Oestridae: Oestrinae	KP954328	KP954359	KP899687	KP899711	KP954390	KP973912	KP235336	KP686280	–
Mesembrinella sp.	Calliphoridae: Mesembrinellinae	KP954342	KP954372	JQ246689[b]	JF439538	KP954404	KP973922	KP235349	KP686293	–
Angioneura abdominalis Reinhard	Calliphoridae: Melanomyiinae	KP954324	–	KP899686	–	KP954386	KP973909	KP235332	KP686276	KP722526
Lucilia sericata (Meigen)	Calliphoridae: Luciliinae	KP954339	KP954369	EU815025	–	KP954401	KP973901	KP235346	KP686290	–
Pollenia pediculata (Macq.)	Calliphoridae: Polleniinae	KP954345	KP954375	FR719179[c]	KP899706	KP954407	KP973925	KP235353	KP686296	KP722544
Melanophora roralis L.	Rhinophoridae	KP954341	KP954371	KP899688	KP899712	KP954403	KP973907	KP235348	KP686292	KP722540
Rhinomorinia sp.	Rhinophoridae	KP954347	KP954377	–	–	KP954409	KP973926	–	KP686298	KP722546
Siphona plusiae Coq.	Tachininae: Siphonini	KP954348	KP954378	KP899664	KP899705	KP954410	KP973927	KP235355	KP686299	KP722547
Epalpus signifier (Walker)	Tachininae: Tachinini	KP954332	KP954363	KP899667	KP899702	KP954394	KP973899	KP235340	KP686284	KP722534
Panzeria ampelus (Walker)	Tachininae: Ernestiini	KP954343	KP954373	KP899674	KP899704	KP954405	KP973908 KP973923	KP235351	KP686294	KP722542
Ceracia dentata (Coq.)	Exoristinae(?): Acemyiini	KP954329	KP954360	KP899676	KP899703	KP954391	KP973913	KP235337	KP686281	KP722531
Winthemia sinuata Reinhard	Exoristinae: Winthemiini	KP954355	KP954385	KP899666[d]	KP899696	KP954417	KP973932	KP235362	KP686306	KP722554
Tachinomyia nigricans Webber	Exoristinae: Exoristini	KP954350	KP954380	KP899672	KP899698	KP954412	KP973896	KP235357	KP686301	KP722549
Blondelia hyphantriae (Tot.)/ *eufitchiae* (Tnsd.)[e]	Exoristinae: Blondeliini	KP954325	KP954356	KP899668	AF364349	KP954387	KP973903 KP973910	KP235333	KP686277	KP722527
Hyphantrophaga virilis (A. & W.)	Exoristinae: Goniini	KP954337	KP954367	KP899673	KP899697	KP954399	KP973919	KP235344	KP686288	KP722537
Lespesia aletiae (Riley)	Exoristinae: Eryciini	KP954338	KP954368	KP899665	KP899695	KP954400	KP973920	KP235345	KP686289	KP722538
Strongygaster triangulifera (Loew)	Tachininae(?): Strongygastrini	KP954349	KP954379	KP899679	–	KP954411	KP973928	KP235356	KP686300	KP722548
Catharosia sp. cf. nebulosa (Coq.)	Phasiinae: Catharosiini	KP954327	KP954358	KP899682	–	KP954389	KP973911	KP235335	KP686279	KP722530
Phasia sp./*Phasia aurulans* Meigen[f]	Phasiinae: Phasiini	KP954344	KP954374	KP899689	KP899701	KP954406		KP235352	KP686295	KP722543
Gymnosoma sp.[g]	Phasiinae: Gymnosomatini	KP954335	GQ409239	KP899683	GQ409462	KP954397	KP973917	KP235343	KP686287	KP722536
Trichopoda pennipes (Fab.)	Phasiinae: Trichopodini	KP954352	KP954382	KP899678	KP899700	KP954414		KP235359	KP686303	KP722551
Cylindromyia binotata (Bigot)	Phasiinae: Cylindromyiini	KP954331	KP954362	KP899677	KP899699	KP954393	KP973906	KP235339	KP686283	KP722533
Euthera setifacies Brooks/ *tentatrix* Loew[h]	Dexiinae(?): Eutherini	KP954334	KP954365	KP899685	–	KP954396	KP973916	KP235342	KP686286	KP722535

(*continued on next page*)

Table 1 (*continued*)

Taxon	Subfamily: Tribe (Family: Subfam.)	18S	28S	COI	EF1α	TPI	CAD	LGL	MCS	MAC
Epigrimyia illinoensis Robertson	Dexiinae: Epigrimyiini	KP954333	KP954364	–	KP899708	KP954395	KP973915	KP235341	KP686285	KP722529
Campylocheta semiothisae (Brooks)	Dexiinae: Campylochetini	KP954326	KP954357	KP899669	KP899691	KP954388	KP973900	KP235334	KP686278	KP722528
Uramya sp.	Dexiinae: Uramyini	KP954353	KP954383	KP899675	KP899694[j]	KP954415	KP973905[i] KP973931	KP235360	KP686304	KP722552
Ptilodexia conjuncta (Wulp)	Dexiinae: Dexiini	KP954346	KP954376	KP899670	KP899690	KP954408	KP973897	KP235354	KP686297	KP722545
Voria ruralis (Fallén)	Dexiinae: Voriini	KP954354	KP954384	KP899671	KP899693	KP954416	KP973902 KP973898	KP235361	KP686305	KP722553
Thelaira americana Brooks	Dexiinae: Thelairini	KP954351	KP954381	KP899684	KP899692	KP954413	KP973904 KP973929	KP235358	KP686302	KP722550

[a] *Cuterebra fontinella* Clark.
[b] *Mesembrinella bicolor* (Fab.), all other sequences are of an undescribed species.
[c] *Pollenia rudis* (Fab.). all other sequences are *P. pediculata*.
[d] *Winthemia rufopicta* (Bigot), all other sequences are *W. sinuata*.
[e] *B. eufitchiae* for EF1α, MAC, MCS, LGL, *B. hyphantriae* for all other genes.
[f] *P. aurulans* for MAC, MCS, LGL, *Phasia aeneoventris* (Williston) for 18S, *Phasia* sp. for all other genes.
[g] *Gymnosoma fuliginosum* Rob.-Des. for 18S, *Gymnosoma nitens* Meigen for 28S and EF1α, all other genes *Gymnosoma par* Walker.
[h] *E. tentatrix* for MAC, MCS, LGL, *E. setifacies* for all other genes.
[i] *Campylocheta plathypenae* (Sabrosky) segment 1 of CAD.
[j] *Uramya pristis* (Walker).

these types of divergence events yet be under adequate selection pressure such that at least some fraction of these changes have been maintained until present time. Three of these genes – molybdenum cofactor sulfurase (MCS), methyl-accepting chemoreceptor (MAC, but see other names), and lethal giant larvae (LGL) – were tapped for use in this study involving reconstruction of relationships within the megadiverse higher fly family Tachinidae and among it and its calyptrate relatives (Supplementary Table 1). These genes, along with CAD, are briefly characterized below.

2.1.1. Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD)

This gene encodes a trifunctional protein associated with the enzymatic activities of the first three enzymes in the 6-step pathway of pyrimidine biosynthesis. CAD, also known as rudimentary in *Drosophila* genetics, was developed and first implemented for use in higher Diptera (Moulton and Wiegmann, 2004, 2007). Use of CAD in insect phylogenetic studies is now widespread. Unfortunately, the most informative region, CAD1 (Moulton and Wiegmann, 2004), is typically not utilized, presumably due to problems with introns. Nonsynonymous variability in CAD region 1 is roughly on par with the following genes, but CAD regions 2–4 are considerably more conserved.

2.1.2. Molybdenum cofactor sulfurase (MCS/MOCOS)

Molybdenum cofactor sulfurase is an enzyme that sulfurates the molybdenum cofactor of xanthine dehydrogenase (XDH) and aldehyde oxidase (AOX1). Sulfation of molybdenum is essential for xanthine dehydrogenase (XDH) and aldehyde oxidase (ADO) enzymes in which molybdenum cofactor is liganded by one oxygen and one sulfur atom in active form. Mutations in MCS are known to cause translucent integument in larval *Bombyx mori* L. (Kômoto et al., 2003). Interestingly, overexpression of *Arabidopsis* molybdenum cofactor sulfurase gene in transformed *Zea mays* L. confers drought tolerance (Lu et al., 2013).

Orthologs of MCS are known from a wide array of insects, including several dipterans, two lepidopterans, a louse, two beetles, and several hymenopterans (Table S1). Intron burden in MCS appears moderate to heavy outside of Diptera. Amino acid divergence among available insect MCS orthologs appears to increase linearly with increasing taxonomic distance. Amino acid divergence between the culicine (Culicinae) mosquitoes *Aedes aegypti* L. and *Culex quinquefasciatus* Say is 28%, whereas that between them and *Anopheles gambiae* Giles (Anophelinae) is 39–40%. Amino acid divergence between these three mosquitoes and *Drosophila melanogaster* Meigen is 52–53%. Divergence between the flies and *Bombyx mori* is only slightly greater at 54–60%, suggesting that backbone functional constraints of the protein are preventing further divergence and that saturation is likely occurring.

2.1.3. Methyl-accepting chemoreceptor (MAC)/Dihydropyridine-sensitive L-type calcium channel

The dihydropyridine-sensitive L-type sodium channels are one of several different kinds of high-voltage-gated calcium channels (HVGCCs) responsible for excitation–contraction coupling of skeletal, smooth, and cardiac muscle and for hormone secretion in endocrine cells. Orthologs are available for several dipterans, two lepidopterans, a beetle, and several hymenopterans (Table S1). Intron burden is moderate to heavy in MAC outside of Diptera and Lepidoptera based on limited availability of orthologs. Amino acid pairwise divergence between the culicine (Culicinae) mosquitoes *Aedes aegypti* and *Culex quinquefasciatus* is 13%, whereas that between *Anopheles gambiae*, *Anopheles darlingi* Root (Anophelinae), *Aedes aegypti* and *Culex quinquefasciatus* ranges from 32% to 40%. Amino acid pairwise divergence between *Drosophila melanogaster* and *Ceratitis capitata* Wied., representing two acalyptrate fly families, is 40%. Amino acid pairwise divergence between the four mosquitoes and *D. melanogaster* ranges from 59% to 66%. The divergence between the flies and *Bombyx mori* is only slightly greater at 65–70%. Based upon these

comparisons saturation of this gene at the amino acid level occurs somewhere near 60%.

2.1.4. Lethal (2) Giant Larvae (LGL)

Lethal (2) giant larvae is a tumor-suppressor gene of *Drosophila* that normally controls cell proliferation and/or differentiation in the optic centers of the brain and the imaginal discs (Jacob et al., 1987; Mechler et al., 1985). Expression of LGL coincides with the two major terminal phases of cell proliferation in the developing fly, i.e., early embryogenesis and late third instar larvae. Reduced expression of Hugl-1, the human homolog of *Drosophila* tumor suppressor gene LGL, contributes to progression of colorectal cancer

Table 2
Primers used in this study. Underlined primers are ones conserved across distantly related taxa. Blue are for amplification, green for re-amplifying.

Region	Primer Name (Lab handle)	Orientation	Sequence (5′ → 3′)[a]	Reference[b]
18S	18SF	Forward	CATATCCGAGGCCCTGTAAT	1
	18SR	Reverse	AGTTTTCCCGTGTTGAGTCA	1
28S	28SF	Forward	CTAACAAGGATTTTCTTAGTAGCGGCGAG	2
	28SR	Reverse	GGTGAGTTGTTACACACTCCTTAGCGGAT	2
COI	LCO 1490	Forward	GGTCAACAAATCATAAAGATATTGG	3
	LEPF1	Forward	ATTCAACCAATCATAAAGATATTGG	4
	LEPR1	Reverse	TAAACTTCTGGATGTCCAAAAAATCA	4
EF1α	efs175 (ef1a-a)	Forward	GGAAATGGGAAAAGGCTCCTTCAAGTAYGC YTGGG	2
	EF2	Reverse	AACTAACGGTGTGACGAGTGTA	2
TPI	M13 tpi 111Fb	Forward	TGTAAAACGACGGCCAGTGGNAAYTGGAAR ATGAAYGG	5
	M13 TpiR275	Reverse	CAGGAAACAGCTATGACGCCCANACNGGYT CRTANGC	5
CAD	54F	Forward	GTNGTNTTYCARACNGGNATGGT	6
	405R	Reverse	GCNGTRTGYTCNGGRTGRAAYTG	6
	320F	Forward	ATHTTYGGNATYTGYYTGGGNCAYCA	6
	SCAD320F	Forward	RTKTTTGGTATTTGYYTGGGTCAYCA	
	338F	Forward	ATGAARTAYGGYAATCGTGGHCAYAA	6
	GLEX P2F	Forward	TGTTTCATGACTTCWCAAAAYC	
	THAX P2F	Forward	GGACGTTGCTTTATGACATCTC	
	680R	Reverse	AANGCRTCNCGNACMACYTCRTAYTC	6
	SCAD680R	Reverse	AARGCATCWCKYACYACYTCGTAYTC	
	Tach P2R	Reverse	GGRTCYAARTTYTCCATRTTRCA	
LGL	P1F1 (36F)	Forward	ACNGCNCARCAYGGYTTYCC	
	P1F2 (38F)	Forward	CARCAYGGNTTYCCNCAYAARCC	
	P2R1 (472RM)	Reverse	CACNCCNGTRCARTCCCARAA	
MAC	P1F (F3)	Forward	GAYGARAARCGNATHATGTAYGARAARCA	
	P1R1 (P13’ INT)	Reverse	GTRGCNATTTGYCGRAARTA	
	P1R2 (tMACP1R)	Reverse	GTRTCYTTYAARTARGCCAT	
	P2F1 (tMAC 1.1F)	Forward	CAYCCNGCNTTYCCNCGYCC	
	P2F2 (tMAC 1.2F)	Forward	CAYCCNGCNTTYCCNCGRCC	
	P2R (tMAC P1R old)	Reverse	TCYTTRTGYGTCARRTGYTCRTG	
	P3F1 (949F)	Forward	ACNACNGARCCRTAYTTAGAYGC	
	P3F2 (957F)	Forward	GCNGGNGGNGCNGGNTAYAT	
	P3R1 (tMAC P2R)	Reverse	TCYTGYTGRCACCAYTCRCA	
	P3R2 (tMAC P2RN)	Reverse	CAYGCYGAYTGYGCRCTRCARAA	
MCS	P1F1 (55FY)	Forward	TGYTAYYTNGAYCAYGCYGG	
	P1F2 (55FR)	Forward	TGYTAYYTNGAYCAYGCRGG	
	P1R	Reverse	ATYAAATCRTTRTARTCRCTRCA	
	P2F	Forward	GGNACNGTNAAYATYGCMATG	
	P2R (840R)	Reverse	CCNGTNSYYTGRTCDATRCADATCAT	

[a] W = A/T; Y = C/T; R = A/G; M = A/C; K = G/T; H = A/T/C; B = C/G/T; N = A/T/C/G.
[b] References for primer sequences are: (1) Kutty et al. (2010), (2) Stireman (2002), (3) Folmer et al. (1994), (4) Hebert et al. (2004), (5) Bertone et al. (2008), (6) Moulton and Wiegmann (2004). Others are newly designed for this study.

(Schimanski et al., 2005). Tumors are produced when both normal LGL alleles are inactivated by either deletion or insertional mutation.

Orthologs of LGL are known from a wide array of insects, including several dipterans, several hymenopterans, a lepidopteran, a beetle, a louse, and an aphid (Table S1). Intron burden is heavy in this gene but within Diptera 5 of the 7 introns typically present occur in the 3′ half of the gene. Slightly over 1400 nucleotides of coding data can be acquired from dipterans using the LGL 36F and LGL 522R primer combination. Use of LGL in non-dipterans might be limited to small fragments from genomic template or cDNA approaches. Amino acid comparisons among available insect orthologs suggest that LGL is the least variant among these three new markers.

2.2. Extraction, amplification, and sequencing of 18S, 28S, COI, EF1α, TPI and CAD

Three legs were removed from fresh or ethanol-preserved flies and stored in ethanol until the extraction procedure. Extractions were performed using the Puregene Tissue Kit (Qiagen Inc.), slightly modifying the manufacturer's protocol. Legs were frozen in liquid nitrogen and pulverized, followed by successive incubation with lysis solution, RNase A, and a protein precipitation solution. The supernatant with DNA was subjected to isopropanol precipitation, centrifugation, ethanol washing, drying, and rehydration of the DNA in 100–200 μL of Qiagen DNA Hydration Solution.

PCR amplification reactions (30 μL) were composed of 13.35 μL deionized H_20, 3 μL of 10× PCR buffer (TaKaRa), 3 μL of 10 mM dNTPs, 4.5 μL of 25 mM $MgCl_2$, 0.15 μL of Taq polymerase (ExTaq, TaKaRa), 1.5 μL of each primer, and 1 μL of DNA solution. Several variations of a touchdown PCR protocol were tried, with the most effective program consisting of an initial denaturation stage of 4 min at 94 °C; 4 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 2 min; 6 cycles of 94 °C for 30 s, 48 °C for 1 min, 72 °C for 2 min; 36 cycles of 94 °C for 30 s, 45 °C for 1 min, 72 °C for 2 min 30 s; and a final extension time of 3 min. Primers used for each gene are listed in Table 2. After visualization on an agarose gel, samples were sent to the University of Arizona Genetics Core (uagc.arl.arizona.edu) for PCR product cleanup, quantification, sequencing reactions, and sequencing. For 28S we sequenced nearly half of the gene, beginning at the 5′ end, for 18S a middle portion of 826 bp, for COI, the 5′ "barcoding" region, and for CAD, regions 1 and 2 of Moulton and Wiegmann, 2004 (see Table 3).

2.3. Amplification and sequencing of MAC, LGL, and MCS

DNA was extracted from three legs or, in some cases, whole specimens using the Fermentas DNA Extraction Kit (Thermo Scientific) and following the manufacturer's protocol. DNA amplifications were performed in 52 μL reactions composed of 36 mL deionized H_20, 5 μL of 10× PCR buffer (TaKaRa), 2.5 μL of 10 mM dNTPs, 1.5 μL of 25 mM $MgCl_2$, 0.2 μL of Taq polymerase (ExTaq, TaKaRa), 3 μL of each primer, and 1–1.5 μL of extracted and purified DNA. The same optimal cycling parameters were used to amplify MAC, LGL, and MCS. The program used a three-step touchdown method with the following settings: 30 s denaturation at 94 °C; 5 cycles of 94 °C for 30 s, 56 °C for 15 s, and 72 °C for 1.5 min; 5 cycles of 94 °C for 30 s, 51 °C for 15 s, and 72 °C for 1.5 min; 30 cycles of 94 °C for 30 s, 46 °C for 15 s, and 72 °C for 1.5 min, and a final extension of 72 °C for 5 min.

The subsequent PCR products were electrophoresed through a 1% agarose gel at 115 V for 25 min, excised from the gel, purified with QiaQuick Gel Extraction kits (Qiagen, Santa Clara, CA) and eluted with 35 μL of elution buffer (10 mM Tris, pH 8.5). Purified PCR products served as templates for sequencing reactions using the same primers used in amplification reactions at 50% concentration. Both strands of each product were sequenced using Big Dye Terminator Cycle Sequencing kits (Applied Biosystems, Carlsbad, California). Resultant products were cleaned using Centri-sep purification columns (Princeton Separations, Adelphia, New Jersey) and sent to the Molecular Biology Resource Facility at the University of Tennessee for sequencing.

2.4. Saturation and informativeness tests

The data were explored by two different means to evaluate the utility of different genes and partitions. First, data from each gene were subjected separately to analysis of phylogenetic informativeness using PhyDesign (http://phydesign.townsend.yale.edu/, López-Giráldez and Townsend, 2011). This approach uses an ultrametric tree and inferred character rates of change to assess probabilities of observing synapomorphies arising at different times on the phylogeny (Townsend, 2007). An ultrametric tree was obtained for input by using the penalized likelihood approach in r8s v.1.8 (Sanderson, 2003; outgroup *Musca* pruned, root depth set to 1.0, smoothing parameter s = 100) to the best tree from the combined RAxML analysis.

Next, distance matrices were generated in PAUP* v.4.10b, both as uncorrected (p) distances and GTR (maximum likelihood) distances, with the rate matrix and gamma shape parameter estimated from the data using the best tree from the combined RAxML analysis. Distances were estimated separately for four gene partitions, based on similar phylogenetic informativeness profiles: (1) CAD + MAC + MCS, (2) TPI + LGL, (3) COI + EF1α, (4) 28S + 18S. For partitions 1–3, distances were estimated separately for first plus second positions and third positions. For the ribosomal partition, distances were estimated separately for hypervariable and remaining regions. A saturation plot of uncorrected verses GTR distances was constructed in R v. 3.0.1 (R Core Team, 2013) and a line representing smoothed means added using the "geom_smooth" command in the package ggplot2 (Wickham, 2009).

2.5. Alignment and analysis

Sequencing output files were assembled and edited using CodonCode Aligner (CodonCode Corp.) (Stireman Lab) or Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, Michigan)

Table 3
Characteristics of included gene fragments.

	18S	28S	COI	EF1α	TPI	LGL	CAD	MAC	MCS
Aligned length	826	1599	688	852	467	1122	1610	2007	1863
# Taxa	33	32	31	28	33	31	33	29	31
# Parsimony informative characters (nucleotides)	41 (5%)	216 (14%)	238 (35%)	231 (27%)	196 (42%)	497 (44%)	711 (44%)	934 (47%)	1035 (56%)
Parsimony informative characters (amino acids)	(NA)	(NA)	32 (14%)	20 (7%)	42 (27%)	100 (27%)	132 (25%)	201 (30%)	301 (48%)
Mean *p* distance (within subfamilies)	0.013	0.044	0.128	0.101	0.14	0.152	0.166	0.156	0.198
Mean *p* distance (between subfamilies)	0.014	0.048	0.134	0.113	0.151	0.165	0.183	0.177	0.224
Mean *p* distance (to outgroup)	0.018	0.047	0.134	0.118	0.17	0.199	0.193	0.213	0.254

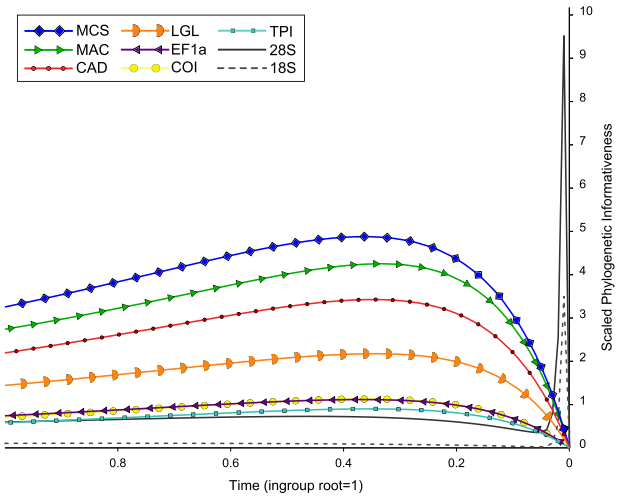

Fig. 1. Net phylogenetic informativeness profiles for each of the nine genes used, generated using PhyDesign (http://phydesign.townsend.yale.edu/, López-Giráldez and Townsend, 2011). The *x*-axis is proportional time from the present (0–1), and the *y*-axis represents the scaled (n/126390.5) sum of the instantaneous asymptotic informativeness of all sites in each gene (see Townsend, 2007).

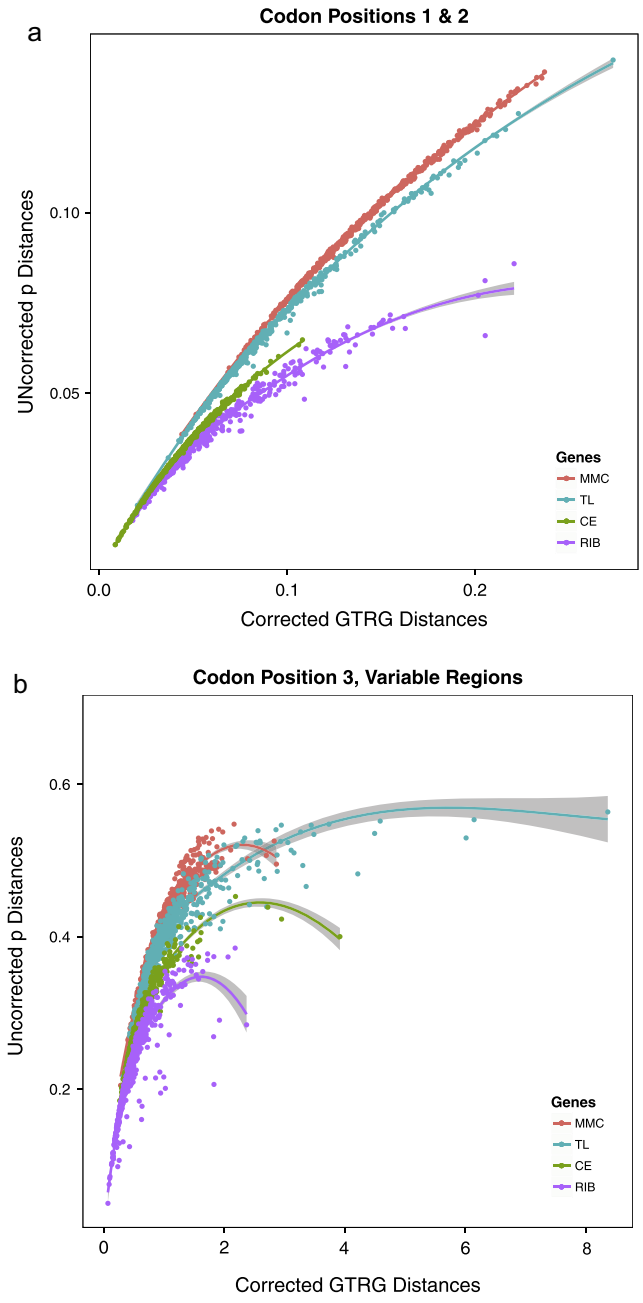

Fig. 2. Saturation plots for four categories of genes used, by codon position. (A) Positions 1 + 2 and (B) position 3. Distances were generated in PAUP* (Swofford, 2003) and plotted in R using the package ggplot2 (Wickham, 2009). MMC = MAC, MCS, CAD; TL = TPI, LGL;CE = COI, EF1α, RIB = 18S, 28S.

(Moulton Lab). Final edited data for 18S and 28S was uploaded to the MAFFT alignment server (http://mafft.cbrc.jp/alignment/server/) and subjected to a secondary structural alignment (Q-INS-I algorithm) using default parameters. Delimitation of hypervariable regions for these genes was performed using gblocks v0.91 (Castresana, 2000) after trying multiple parameter values. The final parameter values used for 28S were 20/32 (minimum sequences for conserved positions), 30/32 (minimum sequences for a flanking position), 3 (maximum contiguous non-conserved positions), 5 (minimum length of a block), and half (allowed gap positions), with respective values for 18S of 17, 28, 8, 5 and half. Data for other genes was aligned by eye in MEGA 5.1 (Tamura et al., 2011) and checked for accuracy as a translated alignment. Neighbor-joining trees were also generated in MEGA for each separately amplified gene partition to check for anomalous results attributed to contamination or mislabeling.

Maximum likelihood analyses of each gene individually, certain combinations of genes, and the total data set were performed using RAxML version 7.4.2 HPC (Stamatakis, 2006) on the Ohio Supercomputer Center Oakley cluster (www.osc.edu). Each data set for individual genes (except 18S, for which a partitioned analysis was not performed) was analyzed twice: unpartitioned, and with third positions (protein-coding genes) or variable-length (hypervariable) loop regions (28S) partitioned separately. Ribosomal genes were also analyzed with hypervariable regions excluded. For each analysis, 100 replicate searches based on random starting trees and the GTRGAMMA model were performed, followed by 1000 bootstrap replicates. A combined analysis with all genes was then performed, partitioned by codon position (position 1 + 2 vs. position 3) and gene class (i.e., protein-coding vs. ribosomal), with hypervariable regions of ribosomal genes also partitioned separately. This analysis was repeated with a GTR + I + G model (in GARLI 2.01 (Zwickl, 2006)) to evaluate effects of a more complex model with a proportion of invariant sites. Additional analyses were performed on a data set consisting of translated amino acid sequences (WAG model with four gamma rate categories, empirical frequencies, ribosomal genes excluded) and with third positions and hypervariable regions excluded (all genes). To compare the effectiveness of different gene categories, the data set was then divided into two separate sets: a "nuc_prot" set of nuclear protein-coding genes (including MAC, MCS, LGL, CAD, and TPI) and a "rib_mit" set with a combination of mitochondrial protein-coding and nuclear ribosomal markers, plus EF1α. EF1α was included with the latter set because informativeness profiles more closely fit with these genes, and because it has often been used in combination with them (e.g., Stireman, 2002). For these last analyses, all nuclear protein-coding genes were included in the same two partitions (third positions separate), while separate partition pairs were created for the ribosomal genes (hypervariable regions separate) and for the mitochondrial COI gene.

A combined Bayesian analysis was performed using MrBayes 3.2.2 (Ronquist et al., 2012). The same four partitions as in the combined likelihood analysis were used. For this analysis, rate variation was modeled with invariant sites plus a gamma shape parameter, and two runs with four chains each were run for a total

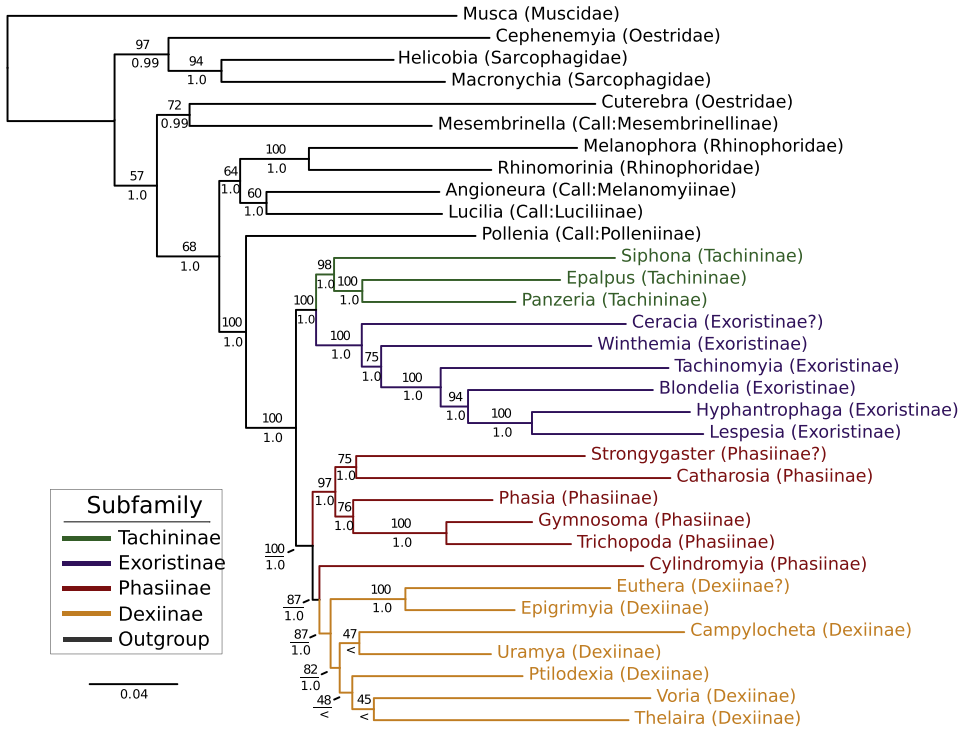

Fig. 3. Maximum likelihood phylogeny from analysis of the combined (nine gene) dataset, generated in RAxML (Stamatakis, 2006). ML bootstrap values and Bayesian posterior probabilities are shown above and below branches, respectively. Colors indicate the four tachinid subfamilies, as indicated in the key. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

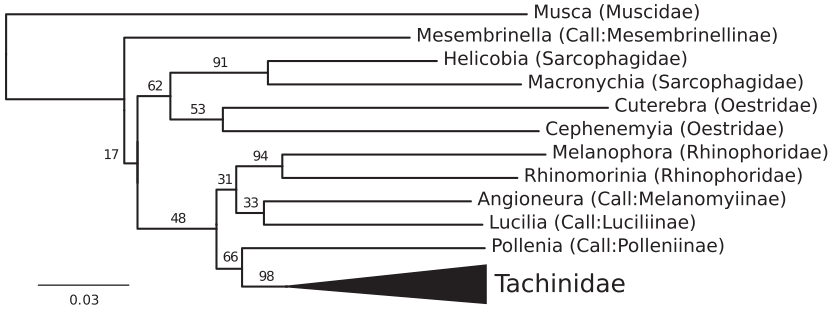

Fig. 4. Maximum likelihood phylogeny from analysis of the combined (seven gene) amino acid data set, showing relationships between outgroups. Note the monophyly of Oestridae, not found in the combined nucleotide analysis.

of ten million generations, after which the convergence statistic (standard deviation of split frequencies) was 0.055. The first 25% of trees were discarded as burn-in.

3. Results

A total of 11,425 aligned positions were obtained, of which 391 were discarded (introns, single-taxon insertions, and ends with low taxon coverage), and 473 were marked as hypervariable regions of ribosomal genes. Alignment lengths, numbers of phylogenetically informative sites, and average pairwise divergence values for each included gene are summarized in Table 3. Genbank accession numbers for these sequences are reported in Table 1.

Analyses of overall phylogenetic informativeness for each gene (Fig. 1) ranked MCS as the most informative, followed by MAC, CAD, and LGL. EF1α, COI, TPI, and 28S all had similar, much lower, informativeness scores across all time scales, while 18S was ranked as least informative. Graphs of per-site informativeness (not shown) resulted in similar rankings, except that TPI (the shortest included gene) ranked much higher, approximately the same as LGL. Saturation curves (Fig. 2a and b) show lack of saturation for positions 1 + 2 and conserved ribosomal regions for all gene sets, while position 3 and hypervariable ribosomal regions show evidence of saturation at the deepest divergences for all gene sets. Sets of genes did differ, however, in their overall degree of divergence, with MAC + MCS + CAD having the highest uncorrected divergence values and ribosomal genes the lowest.

Results of the combined maximum likelihood analysis, shown in Fig. 3, were taken as the best estimate of phylogeny and used to evaluate results from other analyses. Bootstrap (bs) values for most parts of this tree were moderate to high, with the exceptions of relationships within Dexiinae, and some outgroup relationships. The family Tachinidae was recovered as monophyletic in this analysis (bs = 100), as were the tachinid subfamilies Tachininae (bs = 98), Exoristinae (bs = 100) and Dexiinae (bs = 87); Phasiinae exclusive of *Cylindromyia* also formed a strongly supported clade

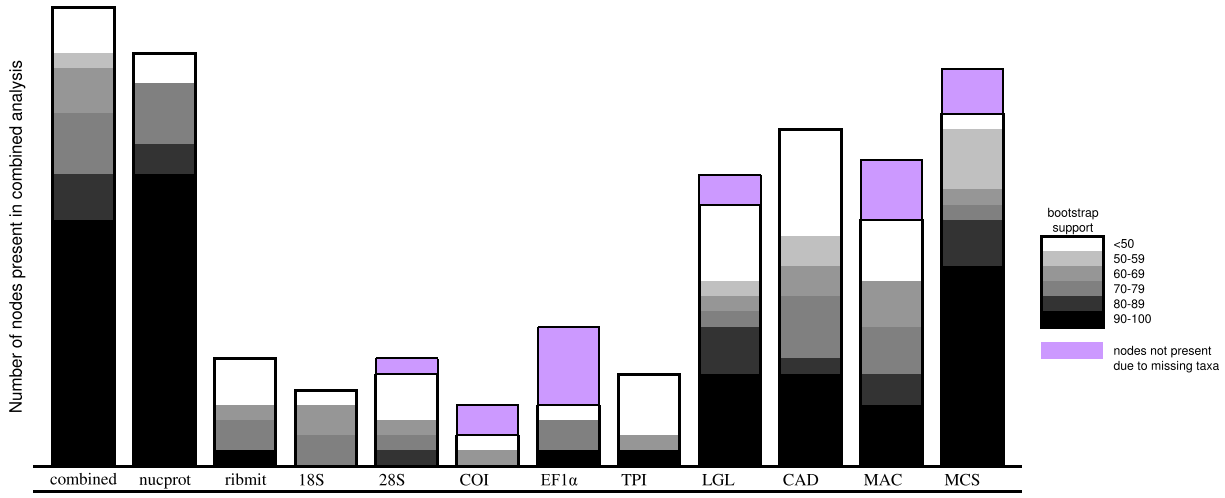

Fig. 5. Concordance of topologies generated by analysis of separate partitions and individual genes, compared to the tree from the combined data set. "nucprot" = nuclear protein-coding genes (except EF1α); "ribmit" = ribosomal and mitochondrial genes, plus EF1α. See also Supplementary Table 2.

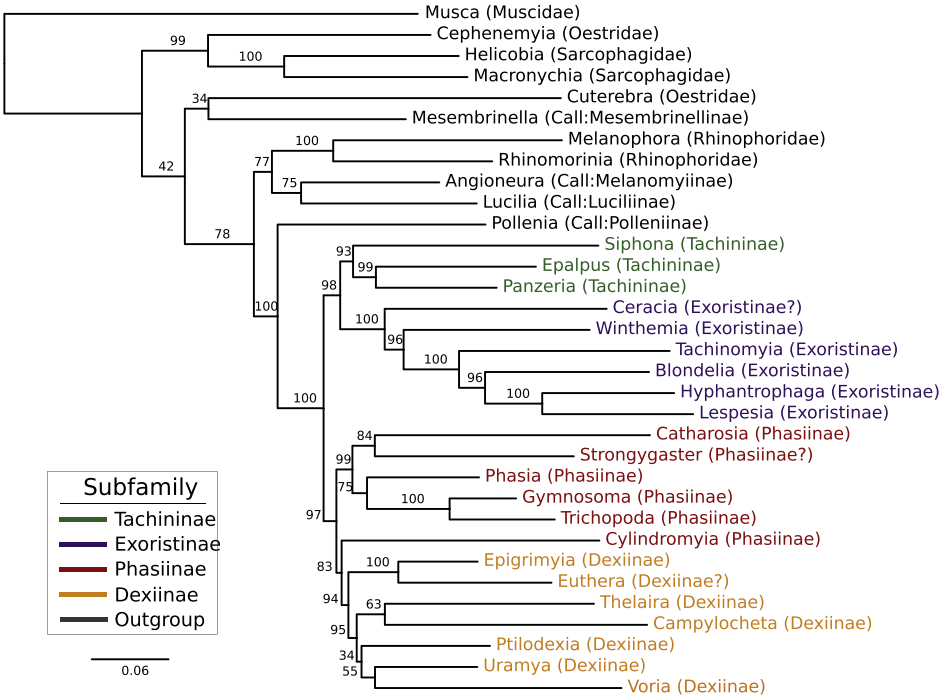

Fig. 6. Maximum likelihood phylogeny from analysis of the "nucprot" data partition, including TPI, LGL, CAD, MAC, and MCS. ML bootstrap values are shown above branches. Colors indicate the four tachinid subfamilies, as indicated in the key. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(bs = 97). *Cylindromyia* was instead found to be sister group to Dexiinae (bs = 87). The three genera of previously uncertain subfamilial placement were each securely placed, with *Euthera* sister to *Epigrimyia* (bs = 100), together sister to remaining Dexiinae (bs = 82); *Ceracia* belonging to Exoristinae (bs = 100), sister to remaining exoristines (bs = 75); and *Strongygaster* nested within Phasiinae, as sister to *Catharosia* (bs = 75). *Pollenia* (Calliphoridae) was strongly supported as the sister group to Tachinidae (bs = 100) in the combined analysis. Rhinophoridae grouped weakly (bs = 64) with remaining Calliphoridae. An unexpected result was the polyphyly of Oestridae, with *Cephenemyia* strongly grouping with Sarcophagidae (bs = 97) and *Cuterebra* with *Mesembrinella* (bs = 72). This result was contradicted, however, by grouping of the two oestrid genera in the amino acid analysis (Fig. 4), as well as in individual partitioned analyses of 28S, EF1α, and TPI (Supplementary Fig. 1b, d and e). The topologies recovered from the combined Bayesian analysis and the GTR + I + G GARLI analysis were identical to the RaxML GTR + G maximum likelihood analysis, except for relationships within Dexiinae: compared to the likelihood GTR + G analysis, the relative positions of *Uramya* and *Thelaira* were switched. Outside of this clade of core Dexiinae, all posterior probability values for the Bayesian analysis were 1.0, with one exception (*Cuterebra* + *Mesembrinella*, 0.99; see Fig. 3).

Results from partitioned analyses of individual genes (shown in Supplemental Fig. 1) varied greatly in degree of support and concordance to the combined analysis (see also Fig. 5; Supplemental Table 2). Results from LGL, CAD, MAC, and MCS each included at least half of the nodes present in the combined analysis

(summarized in Fig. 5). Of these genes, however, MCS did a considerably better job of recovering the combined topology, with moderate to high support for a majority of nodes found in the combined tree (Fig. 5, Supplemental Fig. 1). The remaining genes (18S, 28S, COI, EF1α, TPI) each recovered no more than six nodes present in the combined analysis, with at most one of these having strong support. A few relationships between closely related tribes were more frequently recovered in individual gene trees, including *Lespesia* + *Hyphantrophaga* (28S, COI, TPI, LGL, MAC, MCS), *Trichopoda* + *Gymnosoma* (all genes except COI), *Euthera* + *Epigrimyia* (all genes for which both genera were sampled, i.e., excluding COI and EF1α), and *Epalpus* + *Panzeria* (28S, EF1α, LGL, MAC, MCS). Although resolving most nodes found in the combined analysis, the CAD analysis surprisingly did not resolve two of the above relationships, in each case interposing a single related taxon between the two relevant taxa. In only one case did a relationship not recovered in the combined analysis receive moderate to strong support in an individual gene tree: the MAC analysis grouped *Winthemia* and *Ceracia* together (bs = 79), while the combined analysis placed *Ceracia* as sister to remaining Exoristinae, including *Winthemia*.

Differences between partitioned and unpartitioned analyses for individual genes mostly involved poorly supported nodes and relationships not found in the combined analysis (Supplemental Table S2). Furthermore, for clades that were recovered in the combined analysis, support values for partitioned versus unpartitioned analyses were generally very similar. Among a few exceptions, several scores were notably higher for the partitioned analysis of LGL. Analyses of individual genes excluding third positions (not shown) and hypervariable regions showed lower support and recovered fewer nodes present in the combined analysis.

Splitting the gene data into two separate data sets ("nucprot" and "ribmit") further revealed a strong distinction in utility between the two gene categories. The first category, including only nuclear protein-coding genes (except EF1α), resulted in a well-supported tree identical to the combined analysis, except that *Uramya* and *Thelaira* (Dexiinae) were switched (Fig. 6). Bootstrap values for the "nucprot" tree were generally similar to the combined analysis (Fig. 5, Supplemental Table 2), but showed higher support for a few selected nodes (notably Exoristinae exclusive of *Ceracia*, bs = 96 vs. 75), and much lower support for one, probably erroneous, relationship (*Cuterebra* + *Mesembrinella*, rendering Oestridae polyphyletic; bs ⩽ 50 vs. 72). In contrast, the topology from ribosomal DNA and mitochondrial genes (plus EF1α; Fig. 7) was poorly-supported and recovered only 7 of 30 nodes found by the combined analysis (see Fig. 5). This "ribmit" analysis failed to recover a monophyletic Tachinidae, as well as the subfamilies Exoristinae, Tachininae, and Dexiinae (though Phasiinae exclusive of *Cylindromyia* was recovered), and branches along the backbone of the phylogeny were very short. A closer look at internal versus external branch length distributions for the two trees (Supplemental Fig. 2; adjusted for cross-comparison by dividing by the total tree length) showed that the mean length of internal branches was slightly shorter for the "ribmit" tree. This difference, however, was not statistically different (two-sample Kolmogorov–Smirnov test in R v2.12.0, $p = 0.59$).

4. Discussion

4.1. Phylogenetic Relationships

4.1.1. Oestroid relationships and the phylogeny of the Tachinidae

As mentioned in the introduction, relationships among the major lineages of the Oestroidea remain a major source of contention in the phylogeny of Diptera. Analyses of diversification rates in Diptera have indicated that the proliferation of oestroid and closely related muscoid lineages (Anthomyiidae, Scathophagidae) was associated with a significant increase in diversification rates (Wiegmann et al., 2011). This has made it difficult to resolve relationships among these taxa, particularly with respect to the origin of Tachinidae. Nearly every other family of Oestroidea has been forwarded as a possible sister group of Tachinidae based on morphological and/or molecular evidence (e.g., Kutty et al., 2010; Marinho et al., 2012; McAlpine, 1989; Nirmala et al., 2001; Pape,

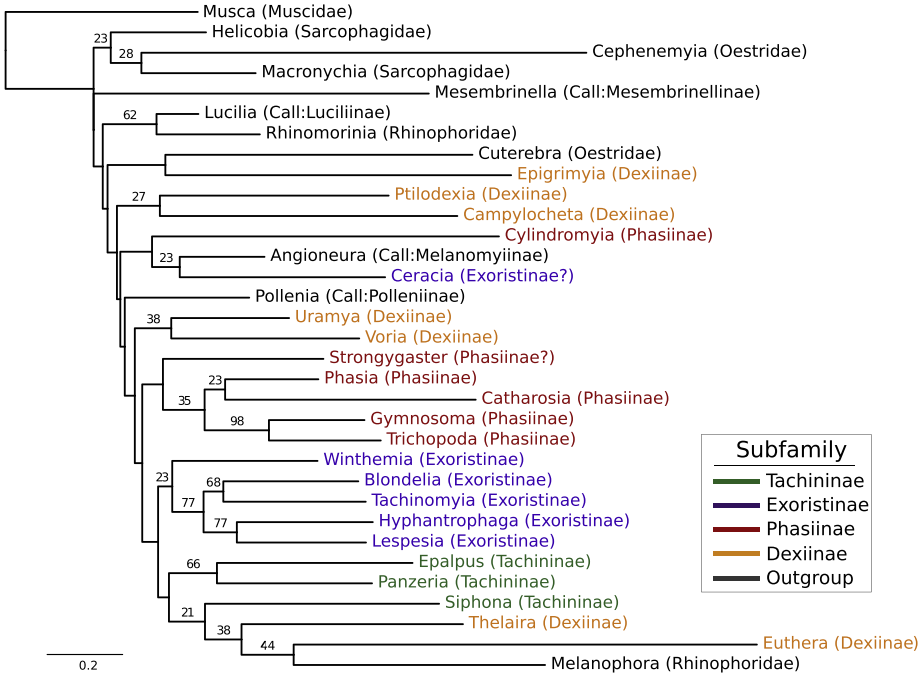

Fig. 7. Maximum likelihood phylogeny from analysis of the "ribmit" data partition, including 18S, 28S, COI, and EF1α. ML bootstrap values are shown above branches. Colors indicate the four tachinid subfamilies, as indicated in the key. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1992; Wiegmann et al., 2011; Zhao et al., 2013). The small and poorly known family Rhinophoridae (woodlouse parasites) has sometimes been proposed as the sister-group to tachinids (e.g., McAlpine, 1989) or included in it (e.g., Sabrosky and Arnaud, 1965), based largely on the shared character states of internal parasitism of arthropods and development of the subscutellum. Both states are less convincing when examined more widely. A convex, sclerotized subscutellum is the most unique and consistent morphological character defining the family Tachinidae, though lacking in at least three unrelated genera (Crosskey, 1984; Wood, 1987). The subscutellum is developed to a lesser degree and partially sclerotized not only in some Rhinophoridae, but also in some members of the calliphorid subfamilies Bengaliinae, Mesembrinellinae and Ameniinae and certain other genera of Sarcophagidae and Oestridae (Rognes, 1997). The parasitoid lifestyle is also not unique to Rhinophoridae and Tachinidae; many Sarcophagidae are parasitoids of insects and other arthropods, and parasitoids of land snails or earthworms are found in the calliphorid subfamilies Ameniinae, Melanomyiinae, and Polleniinae. Although many oestroid lineages were not included in Wiegmann et al.'s (2011) analysis, they found Rhinophoridae (including *Axinia*, now considered a rhinophorid, but nested within Tachinidae in their analysis) and Calliphoridae to be the closest relatives of tachinids. None of these results is individually convincing; taken together they are decidedly less so.

Our results provide strong support (100%) for a close relationship between Tachinidae and the calliphorid subfamily Polleniinae. This relationship was previously recovered in analyses of calliphorid relationships by Nelson et al. (2012), using mitogenomic data, and Singh and Wells (2013), based on four genes, including CAD. Kutty et al. (2010) also found evidence of a close relationship between these two groups in a broader analysis of the Calyptratae (Oestroidea + Muscoidea + Hippoboscoidea) using the same genes, but in their analysis the clade Polleniinae + Mesembrinellinae was reconstructed as sister to Tachinidae, and their single representative of Oestridae (*Cuterebra baeri*) was nested within the Tachinidae. We have yet to identify morphological synapomorphies linking Polleniinae and Tachinidae, but their shared parasitoid habit is at least suggestive of a relationship (though see above). The fact that *Pollenia* use earthworms as hosts has interesting implications for understanding the ancestral hosts of Tachinidae, raising the possibility that they may have been soil or litter dwelling. This view is consistent with the recent suggestion of Cerretti et al. (2014) that the hosts of basal tachinids were not the leaf-chewing lepidopteran larvae that comprise hosts of so many extant tachinids, but rather adult hemimetabolous insects or Coleoptera.

In concordance with previous morphological (Hennig, 1973; Rognes, 1997) and molecular (e.g., Kutty et al., 2010; Marinho et al., 2012; Singh and Wells, 2013) phylogenetic analyses, we do not recover the Calliphoridae (including *Mesembrinella*) as a clade despite our limited sampling. Rather, the four subfamilies (or families) represented are reconstructed as at least three separate lineages interspersed with other oestroid taxa. For combined trees and individual gene trees in which a monophyletic Tachinidae is present, we never recovered a close relationship between *Mesembrinella* and Tachinidae as found by Marinho et al. (2012).

Despite their morphological and biological similarity (Wood, 1987; McAlpine, 1989), our results do not indicate a close affinity between Rhinophoridae (*Melanophora* and *Rhinomorinia*) and Tachinidae. Some single nuclear gene topologies recovered either both rhinophorids (CAD) or *Melanophora* (TPI) as sister to Tachinidae, but support for these relationships was weak (<50%) and contradicted by strongly supported results in the MCS and combined data sets. Instead, Rhinophoridae may be closely related to Calliphoridae, as suggested by Tschorsnig (1985b) and Rognes (1986), but (see Pape and Arnaud, 2001; Rognes, 1997). Similar, more distant relationships between Tachinidae and Rhinophoridae were recovered by Kutty et al. (2010) and Singh and Wells (2013). Our results suggest that the habit of arthropod parasitism evolved independently in the Rhinophoridae and Tachinidae, and in the Sarcophagidae as well.

Our failure to reconstruct a monophyletic Oestridae (*Cuterebra* + *Cephenemyia*) in the combined analysis and the relatively strong support for alternative groupings is troubling. The many morphological synapomorphies of this family (McAlpine, 1989; Pape, 1992, 2001) and their grouping in amino acid analyses (see Results, Fig. 4) lead us to conclude that it does in fact represent a monophyletic group and the results in our combined tree are erroneous. This erroneous placement may be due to the long branches of these two genera and limited sampling of Oestroidea. *Cuterebra* is on the longest branch in our combined tree aside from the muscid outgroup. Our findings support the conclusion of Marinho et al. (2012) and others that the Oestridae are characterized by accelerated rates of both morphological and molecular evolution.

4.1.2. Tachinid subfamilies

It has long been assumed that the Tachinidae are a monophyletic group based on larval and adult characters (see above; Cerretti et al., 2014; Herting, 1960; Wood, 1987) and recent molecular analyses have generally supported this view (e.g., Marinho et al., 2012; Singh and Wells, 2013; Tachi and Shima, 2010; but see Kutty et al., 2010; Wiegmann et al., 2011). However, studies thus far contain few outgroup taxa (e.g., Stireman, 2002), few tachinid taxa (e.g., Marinho et al., 2012) or both (Wiegmann et al., 2011; Zhao et al., 2013), limiting their power to address this issue. Our restricted outgroup sampling also limits the power of our analysis to robustly assess tachinid monophyly, but our results are consistent with this interpretation.

The major division of subfamilies in this study strongly supports that of Cerretti et al.'s (2014) recent analysis using morphological data, grouping Phasiinae + Dexiinae and Exoristi nae + Tachininae. This hypothesis, first proposed by Shima (1989), differs from previous hypotheses grouping subfamilies containing primitively oviparous taxa (Phasiinae + Exoristinae) and those comprised entirely of ovolarviparous taxa (Dexiinae + Tachininae, e.g., Mesnil, 1966). Our combined tree differs from Cerretti et al. (2014) in that the Phasiinae are paraphyletic with respect to the Dexiinae, rather than the reverse, due to the placement of *Cylindromyia* (Fig. 3). Cylindromyiini comprise a highly derived lineage morphologically, but the suggestion that they may not be phasiines, or that the Phasiinae may not be monophyletic is novel. Despite the moderately high (87%) support for the placement of *Cylindromyia*, branch lengths in this region of the tree are quite short, and greater taxon sampling is needed to determine if this result is robust.

We were able to place the enigmatic genus *Strongygaster* with some confidence (97%) within the Phasiinae, where it had been previously placed by Herting (1984) and others (see Blaschke, 2013; O'Hara, 2013). Several recent authors, however, considered the Strongygastrini to be allied with Tachininae (Cerretti, 2010; O'Hara and Wood, 2004). *Strongygaster* is superficially similar to *Phasia* and some other Phasiinae, with large eyes and reduced chaetotaxy, but the biology (ovolarviparity, hosts in four insect orders in addition to Hemiptera) does not match typical Phasiinae, and phallic reduction further obfuscates relationships. Placement of *Strongygaster* within Phasiinae has also been supported by morphological phylogenetic analysis (Cerretti et al., 2014).

Relationships among the Dexiinae are among the most poorly supported in our analyses. The few included taxa are reasonably well supported as a monophyletic group in our combined analysis (87%), but relationships among them are unclear. In analyses of less informative genes (e.g., COI, EF1α) dexiine taxa are widely

dispersed across the tree, whereas with the more informative genes (e.g., MCS, MAC) they are largely clustered but subtended by short branches with low bootstrap support. A clade consisting of the tribes Eutherini and Epigrimyiini, represented here by *Euthera* and *Epigrimyia*, is reconstructed as a basal lineage of Dexiinae in the combined tree. This placement of Eutherini contrasts to previous inclusion in the Phasiinae by several authors (see O'Hara, 2013), but is consistent with classifications by Shima (1989) O'Hara and Wood (2004) and Cerretti (2010). Eutherines appear to be intermediate or "transitional" between the two subfamilies, exhibiting life histories typical of Phasiinae (e.g., Hemiptera hosts), but with ovolarviparity and male genitalia more similar to those of typical Dexiinae.

Monophyly of the subfamily Exoristinae has been supported by all three major phylogenetic analyses of Tachinidae (Stireman, 2002; Tachi and Shima, 2010; Cerretti et al., 2014), and it is strongly supported here as well. Each nuclear protein-coding gene we examined supports Exoristinae as a clade except for EF1α and TPI (Fig. S1). The placement of *Ceracia* at the base of the Exoristinae is also well supported in the combined analysis (Fig. 3), supporting the classification of Herting (1984), and paralleling recent results of Cerretti et al. (2014). *Ceracia* and other Acemyini have the typical grayish, bristly habitus and some other characters typical of Exoristinae, but are highly divergent in genitalic morphology (Tschorsnig (1985a)), host use (Orthoptera), and molecular sequence (Stireman, 2002). Relationships among remaining exoristine tribes are strongly supported and mirror the results of Stireman (2002) and Tachi and Shima (2010). In contrast to the robust recovery of exoristine relationships with molecular data, Cerretti et al. (2014) were unable to achieve resolution within a monophyletic Exoristinae based on morphological characters. Additional taxon sampling will be needed to resolve some outstanding questions, as the present study did not sample the somewhat enigmatic tribes Ethillini, Masiphyini and Euthelairini, or the apparently aberrant blondeliine genera *Phyllophilopsis* and *Trigonospila* (Stireman, 2002; Tachi and Shima, 2010), which may represent distinct lineages within Exoristinae.

Our results reveal little about the monophyly or relationships among the Tachininae due to the very limited representation of taxa. Tachininae are the most morphologically heterogeneous subfamily of Tachinidae, lacking clear morphological synapomorphies and generally acting as a dumping ground for taxa of unknown affinities (Mesnil, 1966). In their recent morphological analysis, Cerretti et al. (2014) found the Tachininae to be polyphyletic, with clades of Myiophasiini + Palpostomatini, Macquartini and Ormiini (none of which is represented here) being placed at the base of Tachinidae and the bulk of the subfamily being paraphyletic with respect to the Exoristinae. We can say little about monophyly and relationships within the Tachininae without denser taxon sampling, although our results do concur with the now widely accepted placement of Siphonini within the Tachininae (see O'Hara, 2013). The other two genera included here, *Panzeria* (Ernestiini) and *Epalpus* (Tachinini), are probably closely related within a large clade ("Tachinini" of Tschorsnig, 1985a) that represents the monophyletic core of Tachininae (Cerretti et al., 2014).

4.2. Phylogenetic utility of genes

Although there remain areas of ambiguity and low confidence, the combination of these nine genes provide a relatively robust outline of major relationships within Tachinidae and between Tachinidae and other oestroid taxa. The signal providing this phylogenetic resolution, however, is unequally distributed among genes, as revealed in analyses of individual genes. As expected, the short barcoding region of COI is not well-suited to recover higher-level relationships, though it did find two nodes within Exoristinae in agreement with the combined analysis. Although mitochondrial genes in general are widely known to show poor characteristics for higher-level studies (Lin and Danforth, 2004), the performance of nuclear ribosomal genes has been mixed in published studies. At higher levels, 28S especially may be very informative; e.g., it featured prominently in the Diptera-wide study of Wiegmann et al. (2011). Some studies (Danforth et al., 2006, Gibson et al., 2011) have found that nuclear ribosomal genes performed on par with nuclear protein-coding genes. Our results, however, are not alone among insect studies in showing a limited contribution of ribosomal genes in multigene datasets (e.g., Almeida and Danforth, 2009; Moulton and Wiegmann, 2007; Petersen et al., 2007; Winterton et al., 2007). Similar poor performance of ribosomal genes has also been found in vertebrates (Makowsky et al., 2010); phylogenetic informativeness profiles of 18S and 28S in the above cited data set were similar to Cox1 (=COI) and were the lowest out of 12 mitochondrial and nuclear genes tested (Moeller and Townsend, 2011).

The nuclear protein-coding genes included here vary widely in their phylogenetic informativeness. Most notably, EF1-α performed poorly, on par with the ribosomal and mitochondrial genes. This gene has been shown in many studies to evolve rapidly, recovering divergence between very closely related species to a degree similar to COI (e.g., Als et al., 2004). Examination of our amino acid alignments of both EF1-α and COI show, however, that this variation is almost entirely due to synonymous substitutions (Table 3). Although appropriate levels of sequence divergence are a key factor affecting gene performance (Makowsky et al., 2010), the distribution and type of substitutions present are also important (Moeller and Townsend, 2011). In particular, synonymous substitutions are much more susceptible to composition bias, which can be a critical problem for recovery of deeper phylogenetic relationships (Jeffroy et al., 2006; Regier and Zwick, 2011).

We found dramatically higher informativeness and accuracy for four included genes: CAD, LGL, MAC, and MCS. Separate analysis of each of these recovered at least half of the nodes found in the combined analysis. These genes, along with TPI, also show the highest proportion of informative characters (>40% of positions informative; Table 3) and, perhaps more importantly, the highest proportion of informative amino acid positions (>25% of positions informative). Length of genes is sure to be a factor as well; TPI's poor performance is undoubtedly affected by its short length in this study (only 457 base pairs included; Table 3), while the four most effective genes all had more than 1,000 included base pairs. These results strongly suggest that amino acid divergence should be a major consideration when choosing markers for a phylogenetic study.

Many phylogenomic and other multigene studies have noted that individual genes seldom recover the optimal topology (e.g., Rokas et al., 2003; Salichos and Rokas, 2013). This finding, translated into the mantra "more is better," represents the main impetus behind the current push towards phylogenomic data. Our results provide some qualified support for this "more is better" approach, in that even most "good" genes, analyzed separately, did not resolve every relationship, and the combination of four "bad" genes resolved (a few) more nodes than each did alone. In this context, the ability of MCS by itself, with generally high support, to recover relationships found in the combined analysis is surprising. Indeed, our results provide rare backing for what could be called the Amy March (one of Louisa May Alcott's *Little Women*) theory of gene sampling: "You don't need scores of suitors [substitute 'genes']. You only need one... if he [it] is the right one." A similar result was found in a much larger study of fungal genes (Aguileta et al., 2008), which found that only 2 of 246 genes tested recovered the combined phylogeny, but these did so with high support values (also see Salichos and Rokas, 2013). Compared to the other genes

(or portions thereof) included here, MCS is long (>1800 base pairs) and more variable at the amino acid level (48% of positions informative; Table 3). Obtaining sequences of MCS for additional tachinid taxa will be a priority for future studies, and we recommend its use for other dipteran taxa as well.

Our results likewise challenge expectations regarding taxon sampling. Although there are many relationships that we cannot test without inclusion of the relevant taxa, our successful resolution of a number of tachinid relationships is surprising given the suspected rapid radiation and huge diversity of tachinids compared to our relatively low taxon sampling (22 ingroup taxa). The generally high support values found here could, however, be questioned based on strong support for one probably incorrect relationship: the grouping of *Cephenemyia* (Oestridae) with Sarcophagidae instead of with the other included oestrid genus (*Cuterebra*). It is known that long-branch attraction and other artifacts can lead to high support for incorrect relationships in genomic-scale datasets (e.g., Jeffroy et al., 2006; Kumar et al., 2012), but it is surprising to see misleading support values in the analysis of a single gene. In this context, our unexpected result regarding the position of *Cylindromyia* should be confirmed with greater taxon sampling, despite the moderately strong support observed.

It is instructive to consider, in hindsight, what our inferred phylogeny of Tachinidae would be without the inclusion of CAD and the other three newly developed nuclear protein-coding genes (Fig. 7). Such a study would have reported a rapid radiation of Oestroidea, resulting in an inability to recover tachinid monophyly or answer major questions about relationships despite nearly 4 kb of sequence data. In contrast, the strongly-supported phylogeny based on the remaining nuclear protein-coding genes (Fig. 6) does not show an especially rapid radiation of Oestroidea, but instead depicts a relatively rapid radiation of tachinid lineages (with the notable exception of exoristine tribes). This comparison serves to highlight an admittedly obvious, though sometimes disregarded, point: inaccurate or unresolved phylogenies cannot give an accurate picture of the temporal course of evolution, just as they cannot answer questions about character evolution.

4.3. Conclusions

A large number of insect studies continue to rely on "traditional" markers (mitochondrial, ribosomal, and some nuclear protein-coding genes) for resolution of difficult, higher-level phylogenetic problems. This is a trend with increasingly predictable results: limited phylogenetic resolution and a limited ability to answer evolutionary questions. Nuclear protein-coding genes are acknowledged to generally require more effort to amplify and sequence, especially when they show more variation in amino acid sequence. Our experience with CAD and the nuclear protein-coding genes introduced here is consistent with this observation. Still, we recommend that insect systematists consider focusing their "time and treasure" on such informative genes that are better suited for addressing questions of higher level phylogenetic relationships. As shown by the Tachinidae, a small number of nuclear protein-coding genes with appropriate levels of amino acid variation can successfully resolve even difficult phylogenetic problems.

The molecular phylogenetic literature is replete with accounts of "rapid radiation" at all taxonomic scales. While this is, of course, an important and widespread phenomenon, it can be tempting to attribute any instance of poor phylogenetic resolution to rapid radiation. In contrast to the story told by "traditional genes," we find that a more selective set of highly informative genes is able to more precisely identify regions of the phylogeny that did experience rapid radiation of lineages, while more accurately depicting their phylogenetic context.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.03.021.

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