Episodic radiations in the fly tree of life

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Flies are one of four superradiations of insects (along with beetles, wasps, and moths) that account for the majority of animal life on Earth. Diptera includes species known for their ubiquity (*Musca domestica* house fly), their role as pests (*Anopheles gambiae* malaria mosquito), and their value as model organisms across the biological sciences (*Drosophila melanogaster*). A resolved phylogeny for flies provides a framework for genomic, developmental, and evolutionary studies by facilitating comparisons across model organisms, yet recent research has suggested that fly relationships have been obscured by multiple episodes of rapid diversification. We provide a phylogenomic estimate of fly relationships based on molecules and morphology from 149 of 157 families, including 30 kb from 14 nuclear loci and complete mitochondrial genomes combined with 371 morphological characters. Multiple analyses show support for traditional groups (Brachycera, Cyclorrhapha, and Schizophora) and corroborate contentious findings, such as the anomalous Deuterophlebiidae as the sister group to all remaining Diptera. Our findings reveal that the closest relatives of the Drosophilidae are highly modified parasites (including the wingless Braulidae) of bees and other insects. Furthermore, we use micro-RNAs to resolve a node with implications for the evolution of embryonic development in Diptera. We demonstrate that flies experienced three episodes of rapid radiation—lower Diptera (220 Ma), lower Brachycera (180 Ma), and Schizophora (65 Ma)—and a number of life history transitions to hematophagy, phytophagy, and parasitism in the history of fly evolution over 260 million y.

molecular systematics | phylogenetics | Insecta | adaptive radiation

The history of life is often portrayed as an ongoing series of evolutionary bursts, with each representing the origin and diversification of unique life forms with different and ecologically significant adaptations. Although the radiations of some groups, such as cichlid fishes of the lakes of East Africa or Darwin's finches, are well documented (1), the big radiations that account for most of the diversity of life on Earth have been more challenging to explore. To understand these radiations, we must resolve the relationships among major taxa, date the origin of these lineages (many of them ancient), and then explicitly consider whether the diversification events are really pulse-like adaptive radiations or, more simply, the result of nonadaptive, or even random, neutral processes.

Although the paradigm of adaptive radiation has been applied to every level of biological classification, the large-scale macroevolutionary pattern expected from ancient repeated episodes of adaptive radiation is unclear. It has been predicted that at this scale, ecologically driven diversification may result in (*i*) significant variation in clade size, uncorrelated to the age of the clade (2), and (*ii*) shifts in average diversification rate coincident with major shifts in morphology, life history, or ecology (3). Another macroevolutionary prediction of repeated adaptive radiation is the widespread existence of paraphyletic series of taxa representing remnants of past radiations as the closest relatives to larger and more recent radiations. Such a pattern has long been recognized as a common feature of the fossil record (4) and has been suspected in the insect order Diptera (true flies) (5). Here, we estimate a unique comprehensive phylogeny of Diptera to reconstruct relationships among families and higher groupings and to identify the origins of major fly radiations.

Although just a few species of flies command most public attention, among them, important pests, such as house flies, horse flies, and mosquitoes, flies actually represent a large part of metazoan diversity. With 152,000 named species and many more unnamed species, flies account for no less than 1 in 10 species on Earth (6). This great fly diversity is traditionally divided into two major groups: the lower Diptera ("Nematocera"), mosquito-like flies with long antennae, and Brachycera, stout and fast-moving flies with short antennae. The majority of species of Brachycera, including *Drosophila* and the house fly, occur in the clade Cyclorrhapha, characterized by their adaptable larval stage (the maggot) and their means of metamorphosis (the puparium). Our understanding of the evolution of flies is obscured by limited and conflicting anatomical and genetic evidence (7) as well as by the difficulty in capturing the enormous species diversity in a single comprehensive phylogenetic analysis. Even well-studied groups of

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flies, such as *Drosophila*, mosquitoes, and house flies, belong to extraordinarily diverse lineages that remain difficult to resolve.

To recover the evolutionary relationships of flies, we divided data collection into two tiers representing alternate sampling strategies so as to maximize both data and taxa: tier 1 includes 42 species across the order sampled for ~30 kb from 14 nuclear genes, full mitochondrial genomes, and 371 morphological features, and tier 2 includes 202 taxa, with at least one species from 95% (149 of ~157) of recognized families, sampled for 5 nuclear genes (7 kb; Table S1). Recent studies have demonstrated that despite missing data, maximizing sequence length for a subsample of taxa outperforms complete matrices with less sequence data (8, 9); thus, we present here the analytical results of the combined tier 1/tier 2 molecular matrix, the largest and most taxonomically broad yet completed for Diptera.

Results and Discussion

Here, we present the most complete phylogeny for flies, and thus reconstruct the evolution of a major branch of the tree of life. In all our tree estimates, we find strong support for traditionally recognized fly lineages and previously undescribed findings that resolve major long-standing issues in fly relationships, including identification of the earliest branching fly lineages, the sister groups to major radiations, and the closest relatives of Drosophilidae. Our trees reveal strong support for the monophyly of Diptera, as well as the existence of a paraphyletic grade of lower Diptera (>52,000 spp., 40 families), and for the monophyly of Brachycera (>100,000 spp., 117 families) and Cyclorrhapha (>64,000 spp., 91 families). Fig. 1 shows our best estimate of fly phylogeny based on a partitioned maximum likelihood (ML) analysis of the combined tier 1/tier 2 molecular data (202 taxa, ranging from 7–37 kb) (Fig. 1 and Fig. S1). We also inferred phylogenetic trees for subsets of these data using an array of model-based methodologies (Fig. S2 and Table S2). Our trees were rooted with taxa from the orders Mecoptera (scorpionflies), Siphonaptera (fleas), and Lepidoptera (moths and butterflies). Recent analyses confirm Mecoptera and Siphonaptera as the closest relatives to Diptera, refuting the hypothesis that the enigmatic two-winged parasites Strepsiptera are close fly relatives (10).

We found the earliest extant fly lineages to be two rare and anatomically bizarre fly families, Deuterophlebiidae and Nymphomyiidae. These fly families include very few species, all of which have extreme morphological adaptations in both larval and adult stages for living in fast-flowing water. These morphological specializations have previously obscured their evolutionary relationships, although a recent molecular analysis hypothesizes the early divergence that we confirm here (11, 12). The remaining lower Diptera is composed of the infraorders Tipulomorpha (crane flies), Culicomorpha (mosquitoes), Psychodomorpha (sand flies), and Bibionomorpha (march flies and gall midges) (11) (Fig. 1). Uncertainty over the relationships among these early fly lineages has made it difficult to determine the origin of Brachycera, but we now find evidence confirming Neodiptera (13) and placing Bibionomorpha as a sister group to Brachycera (14).

Four strongly supported and long-recognized groups are successively nested within Brachycera: Eremoneura (flies with three larval instars), Cyclorrhapha (flies that pupate in a puparium, the hardened skin of the last larval instar), Schizophora (flies that escape from their puparium using an evertable frontal pouch, the ptilinal sac; e.g., *Drosophila*), and Calyptratae (larger flies with wings that have an enlarged basal lobe, the calypter; e.g., house flies). Like that of Brachycera, the sister group of each of these radiations has long been disputed. Counter to current assumptions and prevailing hypotheses (15–17), we find that the families of lower Brachycera ("Orthorrhapha," in part, generally large flower-visiting flies with predatory larvae) form a monophyletic group as the closest relatives of Eremoneura. Our results strongly support that the recently hypothesized (18) North American relict species *Apystomyia elinguis* is the sister to Cyclorrhapha (Fig. 1). The families of lower Cyclorrhapha ("Aschiza") form a paraphyletic grade as expected, but our data support the parasitic family Pipunculidae (big-headed flies) as the closest relatives to Schizophora (Fig. 1), in contrast to morphological findings (19).

Schizophora represents a recent rapid radiation of lineages, including most of the family-level diversity in Diptera (~85 of 157 families, >50,000 spp.). On its own, this radiation is more diverse than that of all terrestrial vertebrates combined. Relationships among schizophorans are poorly supported with the available data (88% of interfamilial relationships with <80 bp), and their robust resolution remains a major challenge for animal phylogenetics. As previously hypothesized (19), Schizophora consists of a paraphyletic grade of acalyptrate flies (including *Drosophila*) and a monophyletic Calyptratae (including house flies). The following acalyptrate superfamilies were recovered with minor modifications: Ephydroidea (including *Drosophila*), Nerioidea, Sciomyzoidea, Lauxanioidea, and Tephritoidea. The relationships of other acalyptrate families remain obscure, likely attributable to the dearth of characters accumulated during the rapid divergence of these early lineages dating from the initial schizophoran radiation. Our combined data suggest a unique placement of Ephydroidea as a sister group to Calyptratae, albeit with low support. Relationships among families of Calyptratae are found to be similar to recently published results (20, 21).

Closest Relatives of Drosophilidae. The unexpected yet robustly supported sister group to Drosophilidae (including *Drosophila*) consists of two enigmatic and highly derived families of insect parasites, Braulidae and Cryptochetidae, within the superfamily Ephydroidea (Fig. 1). In simple terms, the closest relatives of *Drosophila*, the organism that has contributed the most to our understanding of modern genetics, are, rather than being "typical," particularly unusual. Braulidae (bee lice), unrecognizable as flies to the common eye, are tiny, flattened, wingless creatures that live within beehives and attach themselves to adult honey bees. Cryptochetidae are endoparasites of scale insects as larvae and have been used successfully as biocontrol agents (22). The family Drosophilidae itself displays a diversity of life histories, including, like Braulidae, inquilines in honey bee hives and, like Cryptochetidae, parasites of Hemiptera (6). Before this study, there was little agreement on the position of Braulidae and Cryptochetidae within Schizophora, and the undecipherable evolutionary relationships among numerous families of small acalyptrate flies made the identification of a sister group to Drosophilidae challenging. Our dense sampling of family-level lineages provides an opportunity to refine evolutionary comparisons between drosophilids and these newly identified sister groups.

Micro-RNAs Resolve a Contentious Node. The relative placement of the lower cyclorrhaphan families Phoridae (scuttle flies) and Syrphidae (bee mimics known as hover flies) has important implications for understanding evolutionary development within Diptera (23), yet some morphological and molecular analyses yield conflicting phylogenies regarding these two families (24, 25). Although *Episyrphus balteatus* (Syrphidae) is a cyclorrhaphan, it exhibits features of development that are similar to those of other insects; however, unlike other cyclorrhaphans, *Episyrphus* possesses an anterodorsal serosa anlage [middorsal in *Drosophila melanogaster*, *Musca domestica*, and *Megaselia abdita* (Phoridae)], which expresses *hunchback* (absent in *Drosophila*, *Musca*, and *Megaselia*) and exhibits a strong influence of *caudal* on the anteroposterior axis (23), all features observed in noncyclorrhaphan insects. If syrphids are closer relatives to higher flies than phorids, as our molecular tree denotes, it indicates either parallel evolution in *Megaselia* and schizophorans or a reversal to an ancestral mode of development in *Episyrphus*. Fur-

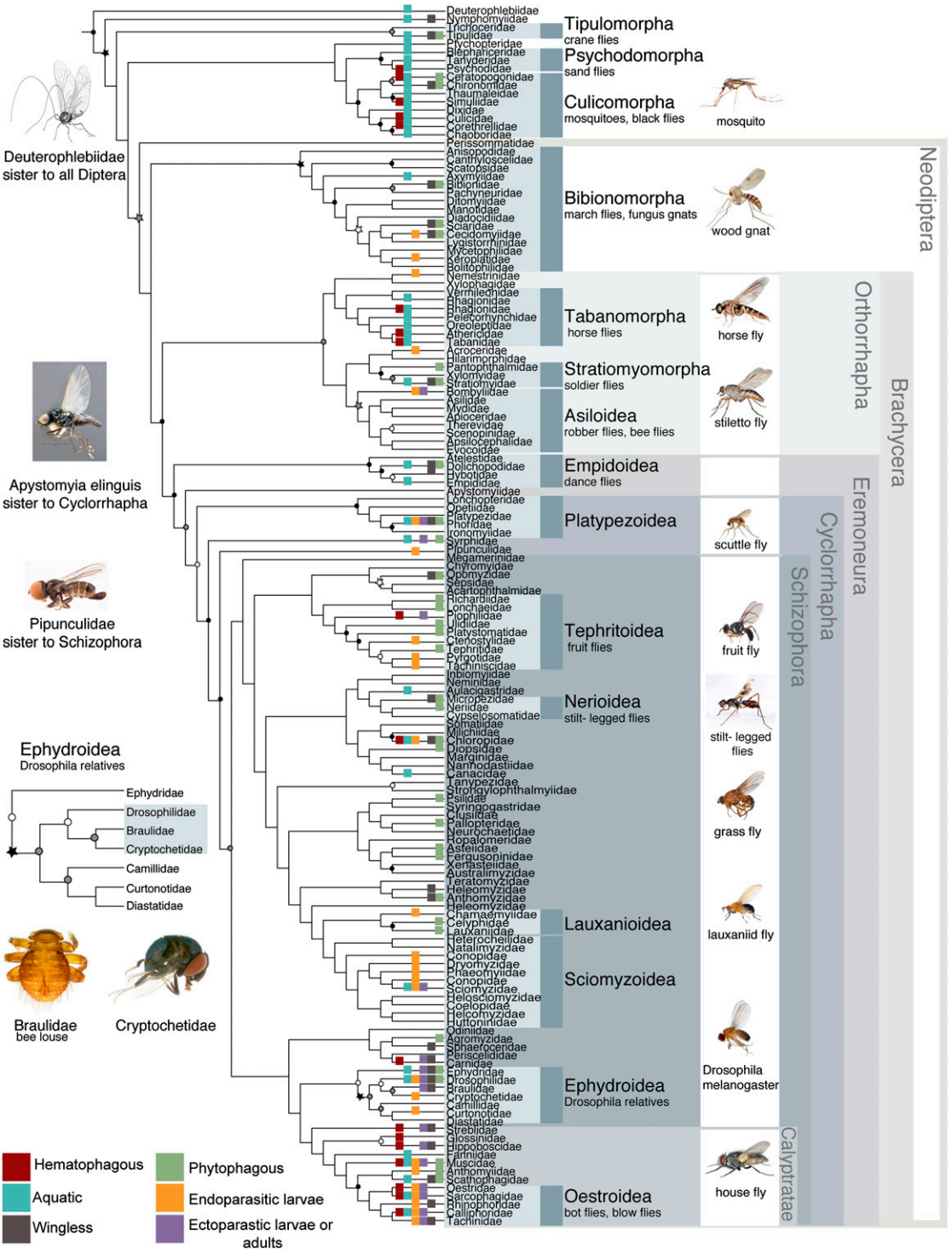

Fig. 1. Combined molecular phylogenetic tree for Diptera. Partitioned ML analysis of combined taxon sets of tier 1 and tier 2 FLYTREE data samples (–lnL = 344155.6169) calculated in RAxML. Circles indicate bootstrap support >80% (black/bp = 95–100%, gray/bp = 88–94%, white/bp = 80–88%). Nodes with improved bootstrap values resulting from postanalysis pruning of unstable taxa are marked by stars (black/bp = 95–100%, gray/bp = 88–94%, white/bp = 80–88%). Colored squares on terminal branches indicate the presence, in at least one species of a family, of ecological traits as shown to lower left. The number of origins of each trait was estimated with reference to the phylogeny, the distribution of each trait among genera within a family, and the known biology of the organisms.

thermore, a paraphyletic relationship of phorids and syrphids would support the hypothesis that their shared special mode of extraembryonic development (dorsal amnion closure) (26) evolved in the stem lineage of Cyclorrhapha and preceded the origin of the schizophoran amnioserosa.

To test this hypothesis, we used a relatively recent phylogenomic marker: small, noncoding, regulatory micro-RNAs (miRNAs). miRNAs exhibit a striking phylogenetic pattern of conservation across the metazoan tree of life, suggesting the accumulation and maintenance of miRNA families throughout organismal evolution

(27). Phylogenetic conservation is also evident in presence/absence comparisons of the expressed miRNA complement in the sequenced genomes of closely related *Drosophila* species (28). We constructed small RNA libraries for two tier 1 taxa, *Episyrphus balteatus* (Syrphidae) and *Megaselia abdita* (Phoridae), and used 454 pyrosequencing. We analyzed the resulting reads with miRMiner (29) to identify known miRNAs and to compare them with known complements of the insects *Tribolium* (Coleoptera) and *Bombyx* (Lepidoptera) and the dipterans *Aedes aegypti*, *Anopheles gambiae*, and *Culex quinquefasciatus* (Culicidae) as well as several *Drosophila* species. The phylogenetic distribution of our newly identified fly miRNAs is concordant with expected fly relationships from our multigene datasets, including the monophyly of Cyclorrhapha and the placement of Syrphidae as a closer relative to Schizophora than Phoridae based on the possession of two unique miRNA families (miR-956 and miR-971) and the possession of a paralogue (miR-318) of the miR-3/309 family and a paralogue (miR-304) of the miR-216/283 family (Fig. 2). Our data support the finding that either *Megaselia* and schizophorans have undergone parallel evolution or *Episyrphus* has undergone loss of several typical cyclorrhaphan developmental features. We also confirm the phylogenetic utility of miRNAs within Diptera.

Divergence Times and Diversification Across the Fly Tree. An important finding from all our analyses is that the phylogenetic history of flies has been episodic. Phylogenetic analyses of fewer taxa (tier 1; Fig. S2) and full taxon sets (tier 1/tier 2; Fig. 1 and Fig. S1) reveal three regions of rapid radiations of fly lineages (Fig. 3), characterized by short branch lengths on reconstructed trees that result from both conflicting and limited evidence assignable to relatively brief periods of recent common ancestry. The identified radiations correspond to the earliest fly lineages (including mosquitos); lower Brachycera (including horse flies); and higher flies, schizophoran Cyclorrhapha (including *Drosophila* and house flies). Our molecular-based time-calibrated phylogeny of dipteran families (Fig. 3, Fig. S3, and Table S3) corroborates the fossil- and molecule-based hypothesis that fly diversification has been driven by episodic bursts of rapid radiation (30). From a Permian origin (31), early aquatic lineages of extant lower Diptera radiated quickly in the Triassic. Later, primarily terrestrial lineages of lower Brachycera, many of which are flower visitors with long proboscides for nectar feeding, radiated in the early Jurassic soon after the recently hypothesized

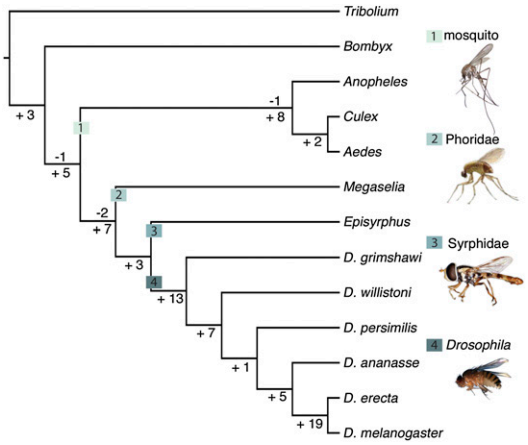

Fig. 2. Phylogenetic distribution of known dipteran miRNA families showing synapomorphic accumulation of miRNA through phylogeny (29). The values below each node are the number of unique miRNAs shown to have been acquired in that lineage, and the numbers above each node are miRNA families that have been lost.

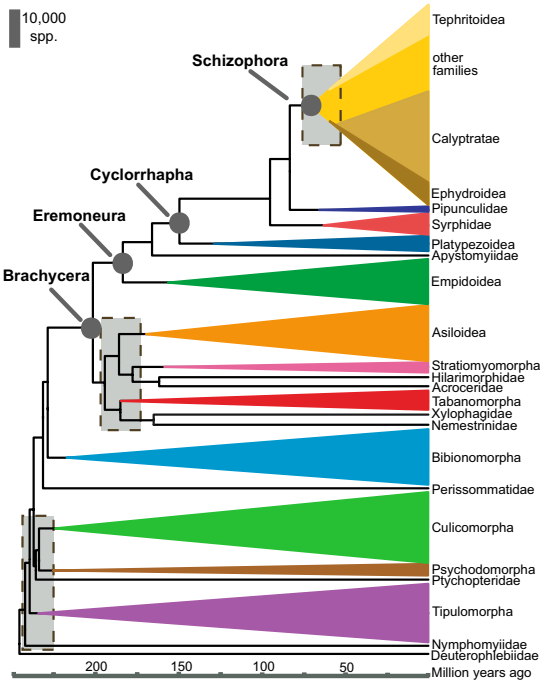

Fig. 3. Chronogram depicting dipteran phylogeny and estimated age of clade divergences. Shaded boxes correspond to areas of rapid radiation and phylogenetic uncertainty. These regions (lower Diptera, lower Brachycera, and Schizophora) are considered major periods of rapid diversification in flies. The vertical height of each triangle represents the approximate number of described species in corresponding clades, with the scale bar indicating 10,000 species.

origin of angiosperms (32). Most recently, we see the radiation of the cyclorrhaphan clade Schizophora. A long gap exists between the earliest known Cyclorrhapha in the Early Cretaceous (*Opetiala*, ~127 Ma) (6) and the earliest confirmed record of the now-dominant Schizophora in the early Paleocene (*Phytomyzites*, 64 Ma) (33). Many acalyptrate families are present in the mid-Eocene Baltic amber deposits (~42 Ma), however (34), implying an explosive radiation of the schizophoran relatives of *Drosophila* in the early Tertiary (65–40 Ma). The schizophoran radiation, which accounts for more than a third of extant fly diversity and 3% of all animal diversity, is (together with macrolepidopteran moths) the largest insect radiation in the Tertiary (6, 9). This vigorous burst of diversification, likely occurring near the K-T boundary, was coincident with the development of the ptilinal sac, an improved escape mechanism for the fly from its puparium.

The diversification of these three predicted rapid radiations, based on likelihood birth–death models, is characterized primarily by very low extinction rates (discussed in ref. 35), with speciation rates actually lower than immediately adjacent lineages (Fig. S3 and Table S4). In other words, flies appear to become diverse by failing to go extinct rather than through the rapid emergence of new forms. This finding is in contrast to other diverse groups that experience patterns of high turnover (3). The schizophoran radiation, in particular, shows strong evidence for accelerated diversification (Table S4), a finding that is extremely robust to details of node calibration and analysis method. Individual fly families, however, exhibit significant variation in diversification rate.

If diversification was constant across groups or through time, a plot of species diversity vs. age would show that older clades are consistently more diverse, both overall and within major taxonomic divisions. This is not the case with flies (Fig. S4). One

reason why diversity and clade age may become decoupled is if diversification slows as old clades saturate available resources, such as might occur in ecologically driven radiation. Moreover, families identified in this plot as having exceptionally low diversification rates are, in many cases, sister groups to major fly clades. Note that the widespread persistence of such "living fossil" lineages is improbable based on most current models of diversification, wherein clade longevity is expected to be strongly correlated with species diversity (36). Taken together, these results suggest an important and complex role for extinction rate variation in generating broad-scale patterns of fly diversity. The generality and macroevolutionary significance of these patterns should be further explored, especially in light of the recognized need for more realistic models to describe diversification at deep phylogenetic levels (37).

Conclusions

Our phylogenetic estimate of Diptera relationships provides an evolutionary framework for future comparative work on species that are critically important to both society and science. Flies originated in wet environments, and as their mechanism for pupation became more impervious to its surroundings (e.g., the cyclorrhaphan puparium) and their larvae more reduced (e.g., the maggot), they adapted to feed in almost any nutrient-rich substrate and have diversified to occupy a broad range of trophic niches. Our phylogeny of flies reveals no fewer than 12 origins of blood feeding (hematophagy), 17 origins of larval endoparasitism (in which fly larvae develop inside the bodies of other organisms), 10 origins of ectoparasitism, 18 losses of functional wings, and 26 origins of plant feeding (38) (Fig. 1). Future work will include understanding the causes of these transitions; whether they are linked causally to changes in speciation and/or extinction rates; and if they are not, understanding what accounts for major fly radiations. Anecdotally, at least some of these trophic and functional shifts are coincident with changes in speciation and/or extinction rates (Fig. 1 and Fig. S3). Nevertheless, evolution within the massive Tertiary radiation of schizophoran flies remains difficult to explain and document, in part because of the complexity of resolving this region of fly phylogeny. We will look to increased sampling of both taxa and sequence data, along with the development of additional genomic character systems, such as miRNAs, to resolve Schizophora in particular but also ancient radiations more generally. The resolution of regions of rapid radiation is a grand challenge that remains for fly phylogenetics—and for the Tree of Life as a whole.

Materials and Methods

Taxon and Gene Sampling. Data collection was completed in two tiers. In tier 1, 42 dipteran taxa and three holometabolous outgroups were sampled for sequence data from 12 nuclear protein-coding genes, 18S and 28S ribosomal DNA, and complete mitochondrial genomes (30 kb; Table S1). In tier 2, 202 taxa, comprising at least one species from 149 of the ~157 recognized families and including three additional outgroups, were sampled for 5 nuclear genes (7 kb; Table S1). Laboratory procedures for sequence collection of nuclear genes and mitochondrial genomes can be found in *SI Materials and Methods*.

Morphology. From numerous studies, we established a list of 457 putatively informative morphological characters to cover the anatomical diversity of Diptera. Team consultation reduced this to 371 external and internal morphological characters for larvae (93), pupae (11), and adults (267, including 55 head, 54 wing, 31 female genitalia, and 49 male genitalia). We scored 42 tier 1 dipteran taxa and 5 holometabolous outgroup taxa. More information regarding the collection of morphological data can be found in *SI Materials and Methods*.

Phylogenetic Inference. Likelihood (using RAxML) (39) and Bayesian (using MrBayes) (40, 41) analyses were completed using variations of taxon sampling and character inclusion and partitioning, the results of which can be found in Figs. S1 and S2 and Table S2. For the ML analysis shown in Fig. 1, molecular data from tier 1 and tier 2 were combined (202 taxa, 7–30 kb); ribosomal, mitochondrial, and nuclear protein-coding genes were partitioned separately; and third positions and regions of ambiguous homology were excluded. In RAxML, 1,000 independent runs from random starting trees were performed to find the highest scoring replicate (9). ML node support was calculated by acquiring bootstrap values from heuristic searches of 1,000 resampled datasets, using the rapid bootstrap feature of RAxML (42). Taxa were tested for instability using a custom R script, and unstable taxa were removed to observe the effects on support values. More information regarding phylogenetic analyses can be found in *SI Materials and Methods*.

miRNA Analyses. We constructed miRNA libraries for two fly species from the dataset of tier 1, *Episyrphus balteatus* (Syrphidae) and *Megaselia abdita* (Phoridae). Libraries were constructed as described by Wheeler et al. (29). The small RNA libraries were sequenced using the Roche GS-FLX pyrosequencer of the North Carolina State University Genome Sciences Laboratory, and the resulting sequences were analyzed with miRMiner (29). In all, we identified 137,059 miRNA sequences in our sample that were assignable to 1 of 159 known miRNA families.

Divergence Times and Diversification Analyses. Divergence time estimates were calculated for the ingroup-only combined dataset using the penalized likelihood method (43) in r8s, version 1.71, with fossil constraints included. Diversification analyses were completed using the MEDUSA program (3). Additional details regarding diversification analyses can be found in *SI Materials and Methods*, Figs. S3 and S4, and Table S3 and Table S4.

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