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The phylogeny and evolution of host choice in the Hippoboscoidea (Diptera) as reconstructed using four molecular markers

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Abstract

Hippoboscoidea is a superfamily of Diptera that contains the Glossinidae or tsetse flies, the Hippoboscidae or louse flies, and two families of bat flies, the Streblidae and the Nycteribiidae. We reconstruct the phylogenetic relationships within Hippoboscoidea using maximum parsimony and Bayesian methods based on nucleotide sequences from fragments of four genes: nuclear 28S ribosomal DNA and the CPSase domain of CAD, and mitochondrial 16S rDNA and cytochrome oxidase I. We recover monophyly for most of the presently recognized groups within Hippoboscoidea including the superfamily as a whole, the Hippoboscidae, the Nycteribiidae, the bat flies, and the Pupipara (=Hippoboscidae+Nycteribiidae+Streblidae), as well as several subfamilies within the constituent families. Streblidae appear to be paraphyletic. Our phylogenetic hypothesis is well supported and decisive in that most competing topological hypotheses for the Hippoboscoidea require significantly longer trees. We confirm a single shift from a free-living fly to a blood-feeding ectoparasite of vertebrates and demonstrate that at least two host shifts from mammals to birds have occurred. Wings have been repeatedly lost, but never regained. The hippoboscoid ancestor also evolved adenotrophic viviparity and our cladogram is consistent with a gradual reduction in the motility of the deposited final instar larvae from active burrowing in the soil to true pupiparity where adult females glue the puparium within the confines of bat roosts.

Keywords: Hippoboscoidea; Wing loss; Ectoparasitism; Evolution; Phylogeny; CAD

1. Introduction

Hippoboscoidea are highly specialized ectoparasitic flies with four recognized family-level taxa: Glossinidae, Hippoboscidae, Streblidae, and Nycteribiidae (Hennig, 1973; McAlpine, 1989; but see Griffiths, 1972). The well-known Glossinidae (tsetse flies) are free-living and only come into close contact with their host during feeding. The other three families, Hippoboscidae, Nycteribiidae, and Streblidae, are all genuine ectoparasites (i.e., species with a trophic and a spatial association to host) spending all or most of their adult life within the fur or among the feathers of their mammal and bird hosts. These families exhibit a large number of unique and striking morphological and physiological adaptations, most of which are specifically associated with their ectoparasitic lifestyle. One of the most remarkable of these is adenotrophic viviparity (Meier et al., 1999). The larvae develop individually in the female oviduct, where they are fed by secretions from accessory glands. The fully mature 3rd instar larva is deposited either as a motile larva, which quickly pupates within its last larval skin (Glossinidae, Hippoboscidae), or as a more or less soft pre-puparium (Streblidae, Nycteribiidae). At the time of deposition, the weight of the larva can exceed the weight of the female (Hill, 1963).

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Although the group has received considerable taxonomic attention, comparatively little is known about the relationships among the families. As a consequence, phylogenetic assessments of the evolution of host choice have not yet been possible and much of the literature on the subject is highly speculative. A recently published molecular systematic analysis by Dittmar et al. (2006) addressed some of these problems, but it focused largely on the relationships within Streblidae and Nycteribiidae and included only a few species from the remaining families. Here, we present results from a complementary phylogenetic study that includes a broader taxon sample from the Hippoboscidae and Glossinidae and we explore the use of different genetic markers than those used in Dittmar et al. (2006). In addition to the mitochondrial 16S rDNA used by Dittmar et al. (2006), we sequenced fragments of the nuclear genes 28S ribosomal DNA (28S rDNA), the carbamoylphosphate synthase (CPSase) domain of CAD (Moulton and Wiegmann, 2004), and the mitochondrial gene cytochrome oxidase I (COI), for 35 species. The goal of the current study was to test the monophyly of the Hippoboscoidea, test the monophyly of the four subordinate families, clarify the phylogenetic relationships among the families, and to use the resulting trees to reconstruct key events in the evolution of Hippoboscoidea.

1.1. Family portraits: biology and systematics

Nycteribiidae are obligate ectoparasites of bats with highly specialized and reduced adult morphology. The wings are completely reduced, the thorax is dorsoventrally flattened, the legs are inserted dorsally, and the head is folded backwards resting on the thorax. The flies thus have a spider-like appearance and are regularly delivered to spider taxonomists for identification (N. Scharff, pers. comm.). The adults spend all of their life in the fur of the host, leaving the host only for brief periods in order to affix a puparium to the wall or ceiling of the bat roost. Numerous morphological synapomorphies have left little doubt that the Nycteribiidae are monophyletic (Hennig, 1973). Three subfamilies are recognized: the Archinycteribiinae and Cyclopodiinae (on Megachiroptera), and the Nycteribiinae (on Microchiroptera; Hennig, 1973; Theodor, 1967). Morphological support for this subdivision comes mainly from the number of tergites on the female abdomen, the position of the thoracic sutures and setae, shape of tibiae, and overall chaetotaxy. However, these characters are highly variable (Theodor, 1967) and currently the most consistent character is host choice, although behavioral features such as host use may be particularly prone to convergence (Blomberg et al., 2003). Furthermore, character polarity is unknown; i.e., it is unclear whether the Archinycteribiinae+Cyclopodiinae (Megachiroptera) or the Nycteribiinae (Microchiroptera) are based on a plesiomorphic host association.

The Streblidae are also obligate bat-ectoparasites, but unlike the Nycteribiidae most species retain fully functional wings for at least part of their life. An exception is *Ascodipteron* Adensamer 1896 in which females, after mating, embed themselves in the tissue of the host. Wings and legs are shed and the fly attains a sack- or flask-like appearance while the males retain their wings throughout life. The morphology of the remaining Streblidae is also unusually variable. For example, some species are dorsoventrally flattened, while the Nycterophiliinae are laterally flattened. Because of these unique traits, finding support for streblid monophyly has been difficult (McAlpine, 1989). Most autapomorphies proposed by McAlpine (1989) are invalid because they are based on wing morphology and thus inapplicable for the other family of bat flies (Nycteribiidae); i.e., it remains unclear whether these features are autapomorphic for Streblidae or Streblidae+Nycteribiidae. Similarly problematic are McAlpine's (1989) characters pertaining to thorax morphology because the nycteribiid thorax is so highly modified that homologies are difficult to establish. McAlpine (1989) also listed the absence of spermathecae as a streblid synapomorphy although Wenzel and Peterson (1987) considered the spermathecae "probably present". This conflict may be due to the fact that several hippoboscoid families have unsclerotized spermathecae (Maa and Peterson, 1987; Peterson and Wenzel, 1987; Wenzel and Peterson, 1987), thus making the feature very difficult to identify in, for example, pinned specimens. Currently, the Streblidae is subdivided into five subfamily-level taxa (McAlpine, 1989, but see Hennig, 1973). The Nycteriboscinae and the endoparasitic Ascodipterinae are restricted to the Old World, while the Trichobiinae, the Nycterophiliinae, and the Streblinae are found only in the New World.

The Hippoboscidae contains approximately 150 species that infest the plumage of various birds or the fur of mammals. However, whether the ancestral host species was a bird or a mammal remains unknown. Bequaert (1954) maintained that the hippoboscid ancestor was a bird parasite, although he admitted that there was little evidence to support this hypothesis. In contrast, in an earlier publication Hennig (1965), assuming a sister group relationship between the Hippoboscidae and the Glossinidae, asserted that the most likely ancestral host of the Hippoboscidae was a mammal. The Hippoboscidae are dorsoventrally flattened, and, in contrast to the Nycteribiidae, the head is prognathous and broadly confluent with the thorax. Overall, these flies have a crab-like appearance, although they are generally called "louse flies". Most species have fully developed and functional wings, but some are stenopterous and a few apterous. Despite the morphological variability, the monophyly of the Hippoboscidae has been almost universally accepted (Bequaert, 1954; Hennig, 1973; McAlpine, 1989). The family is subdivided into three subfamilies (Maa and Peterson, 1987). The Lipopteninae are restricted to mammals while the Ornithomyiinae and Hippoboscinae parasitize both mammals and birds. However, the sole member of the Hippoboscinae known to infest birds is *Struthiobosca struthionis* (Janson, 1889)

which is only found on ostriches, the ecologically most mammal-like bird.

The fourth family of Hippoboscoidea, the Glossinidae, is not only the least speciose (22 spp.), but it is also by far the most familiar due to its notoriety as the vector for *Trypanosoma* parasites that cause sleeping sickness in humans and nagana in livestock (Krinsky, 2002). Morphologically it is the least modified hippoboscoid family. The adults are free-living and only come in contact with the host during feeding. Thus, apart from their proboscis, the glossinids look like typical calyptrate flies and do not display any of the spectacular adaptations for ectoparasitism that are so common in the Hippoboscidae, Nycteribiidae and Streblidae. While retaining many ancestral calyptrate features, the monophyly of the Glossinidae was accepted by Hennig (1973) and supported by a number of synapomorphies in McAlpine (McAlpine, 1989: e.g., arista with long plumules on dorsal surface, very elongated palpi, and proboscis).

1.1.1. Hippoboscoidea phylogenetics

There are relatively few issues in hippoboscoid phylogeny and classification that are not contentious. Most authors agree that the Nycteribiidae and the Hippoboscoidea are well supported monophyletic groups. The latter is supported by two cladistic analyses using DNA sequence data (Nirmala et al., 2001; Dittmar et al., 2006) and several morphological synapomorphies including, adult mouthparts that are uniquely modified for hematophagy, and adenotrophic viviparity with the deposition of mature 3rd instar larvae (Hennig, 1973; McAlpine, 1989). Arriving at the modern concept of Hippoboscoidea, however, was a slow process. Initially, the Hippoboscoidea only included the three core families that are today known as "Pupipara" (Hippoboscidae, Nycteribiidae, Streblidae). The monophyly of this grouping was initially controversial (e.g., Bequaert, 1954; Falcoz, 1926; Hendel, 1936; Jobling, 1929; Lameere, 1906; Müggenburg, 1892; Muir, 1912), but is now defended by many authors (e.g., Griffiths, 1972; Hennig, 1973) and sometimes the Nycteribiidae and Streblidae are even included within the Hippoboscidae (Biosystematic World Database of Diptera: <http://www.sel.barc.usda.gov/diptera/names/BDWDabou.htm> Griffiths, 1972). The Hippoboscoidea in its present composition including the Glossinidae and Pupipara was only proposed in 1971 by Hennig (as "Glossinoidea") although Speiser had already pointed out similarities in 1908.

Controversial are most other Hippoboscoidea relationships. For example, its position within the dipteran clade Calyptratae is not certain (see Bequaert, 1954). The current consensus is that the superfamily is probably the sister group to all remaining Calyptratae (Hennig, 1973; McAlpine, 1989; Nirmala et al., 2001), but previously the most common placement was close to or within the Muscidae (Bequaert, 1954; Muir, 1912), or even as the sister group of the Oestridae (Lameere, 1906; Pollock, 1971, 1973; but see Griffiths, 1976). To begin to address the placement of Hippoboscoidea within the Calyptratae, we include six species from four other calyptrate families as outgroups.

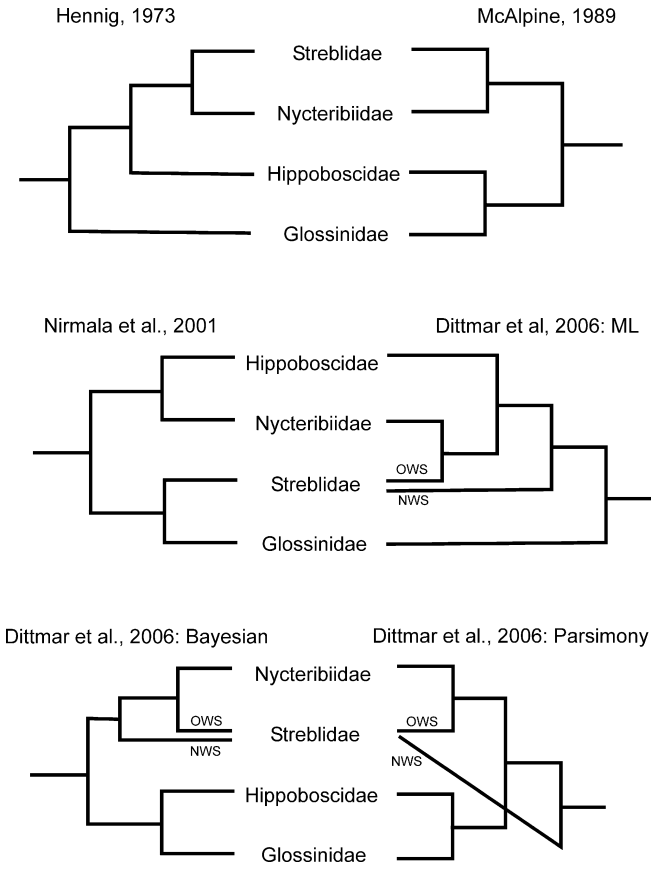

Fig. 1. Previously proposed phylogenetic relationships within the Hippoboscoidea. OWS, Old World Streblidae; NWS, New World Streblidae.

Similarly unclear are the interfamiliar relationships in Hippoboscoidea which hinder the reconstruction of key events in the evolution of this group. Hennig (1973) proposed that genuine ectoparasitism evolved once and that the two families of bat parasites form a monophyletic group (Fig. 1), while McAlpine (1989) favored a sister group relationship between Glossinidae+Hippoboscidae and Streblidae+Nycteribiidae. The latter view was also supported by Nirmala et al.'s (2001) molecular data and Dittmar et al.'s (2006) maximum likelihood analysis with the exception that the Streblidae was paraphyletic (Fig. 1). Streblid paraphyly was also found in Dittmar et al.'s (2006) parsimony and Bayesian likelihood analyses which otherwise yielded again very different trees for suprafamiliar relationships (Fig. 1).

2. Materials and methods

2.1. Taxon sampling, DNA extraction and sequencing

Thirty species representing 7 out of 10 subfamilies of Hippoboscoidea are included in our analysis. Specimen

data are given in Table 1 and voucher specimens are deposited in the collection of the North Carolina State University. Due to uncertainty with regard to the placement of the Hippoboscoidea within the Calyptratae, we included six calyptrate outgroups from four families and the acalyptrate, *Drosophila melanogaster* Meigen 1830, for rooting the tree.

DNA was either extracted from single legs using the DNAeasy kit (Qiagen, Santa Clara, CA) following the manufacturer's protocol, except that the elution volume was 30 µl, or in some cases we punctured the specimen and used a CTAB phenol/chloroform extraction protocol keeping the exoskeleton of the specimen as a voucher. A 760 bp fragment of the carbamolyphosphate synthetase (CPS) region of the CAD gene (also know as "rudimentary" in Diptera) was amplified and sequenced according to the protocol described by Moulton and Wiegmann (2004). Initial amplification was carried out with the primers 787F and 1124R (Table 2). For reamplification and subsequent sequencing the primers 806F and 1098R were used. Approximately 2 kb of the 28S rDNA were amplified in three overlapping pieces using the primers listed in Table 2 and the PCR conditions given in Collins and Wiegmann (2002). Partial sequences for COI and 16S were obtained using the primers specified in Table 2 under standard conditions (Savage et al., 2004).

For sequence editing and contig construction we used Sequencher 4.2 (Gene Codes Corporation Inc, Ann Arbor, Michigan, USA). COI and CAD were unambiguously aligned by eye using MacClade 4.03 (Maddison and Maddison, 2001) and neither displayed stop codons when translated to amino acid sequence. The sequences for 16S and 28S were aligned in ClustalX (Thompson et al., 1997) using the following settings: gap opening cost (10) and gap extension cost (0.20). We subsequently performed minor manual adjustments in MacClade 4.03 (Maddison and Maddison, 2001). Alignments are available on request, and GenBank Accession Nos. are given in Table 1. Leading and trailing gaps were treated as missing data, while internal gaps were scored as a 5th character state.

Table 1
Species information and GenBank Accession Numbers

Taxon	Family	Host	CAD	COI	16s	28s
Hydrophoria lancifer (Harris, 1780)	Anthomyiidae	Not ectoparasitic	EF531164	EF531194	n.a.	EF531129
Drosophila melanogaster Meigen, 1830	Drosophilidae	Not ectoparasitic	NM206765	NC001709	AJ400907	CP000170
Fannia canicularis (Linnaeus, 1761)	Fanniidae	Not ectoparasitic	EF531184	EF531197	n.a.	EF531132
Muscina stabulans (Fallen, 1817)	Muscidae	Not ectoparasitic	EF531167	EF531210	EF531117	EF531145
Stomoxys calcitrans (Linnaeus, 1758)	Muscidae	Not ectoparasitic	EF531173	EF531216	EF531122	EF531151
Cordilura ciliata (Meigen, 1826)	Scathophagidae	Not ectoparasitic	EF531159	EF531195	n.a.	EF531130
Glossina austeni Newstead, 1912	Glossinidae	Mammals	EF531177	EF531198	EF531108	EF531133
Glossina brevipalpis Newstead, 1910	Glossinidae	Mammals	EF531166	EF531199	EF531109	EF531134
Glossina fuscipes Newstead, 1910	Glossinidae	Reptiles, mammals	n.a.	EF531226	EF531126	n.a.
Glossina morsitans Westwood, 1851	Glossinidae	Mammals	EF531178	EF531200	AF072373	EF531135
Glossina pallidipes Austen, 1903	Glossinidae	Mammals	EF531179	EF531201	EF531111	EF531136
Glossina palpalis (Robineau-Desvoidy, 1830)	Glossinidae	Reptiles, mammals	EF531180	EF531202	EF531112	EF531137
Glossina swynnertoni Austen, 1923	Glossinidae	Mammals	EF531181	EF531203	EF531113	EF531138
Crataerina pallida (Latreille, 1811)	Hippoboscidae	Mammals	EF531165	EF531196	EF531107	EF531131
Hippobosca equina Linnaeus, 1758	Hippoboscidae	Mammals	EF531189	EF531208	EF531116	EF531143
Hippobosca rufipes Olfers, 1816	Hippoboscidae	Mammals	EF531188	EF531207	EF531115	EF531142
Lipoptena cervi (Linnaeus, 1758)	Hippoboscidae	Mammals	EF531185	EF531204	EF531114	EF531139
Lipoptena depressa (Say, 1823)	Hippoboscidae	Mammals	EF531187	EF531206	n.a.	EF531141
Lipoptena mazamae Rondani, 1878	Hippoboscidae	Mammals	EF531186	EF531205	n.a.	EF531140
Melophagus ovinus (Linnaeus, 1758)	Hippoboscidae	Mammals	EF531190	EF531209	EF531104	EF531144
Ornithoctona sp. Speiser, 1902	Hippoboscidae	Birds	n.a.	EF531223	n.a.	EF531158
Ornithomya anchineura Speiser, 1902	Hippoboscidae	Birds	n.a.	EF531227	n.a.	n.a.
Ornithomya avicularia (Linnaeus, 1758)	Hippoboscidae	Birds	EF531168	EF531211	EF531118	EF531146
Ornithomya biloba (Dufour, 1827)	Hippoboscidae	Birds	EF531169	EF531212	EF531119	EF531147
Ornithomya chloropus (Bergroth, 1901)	Hippoboscidae	Birds	EF531170	EF531213	EF531120	EF531148
Ornithomya fringillina (Curtis, 1836)	Hippoboscidae	Birds	EF531171	EF531214		EF531149
Ortholfersia minuta Paramonov, 1954	Hippoboscidae	Mammals	EF531182	EF531221	EF531123	EF531156
Pseudolynchia canariensis (Macquart, 1840)	Hippoboscidae	Birds	EF531162	EF531220	EF531103	EF531155
Stenepteryx hirundinis (Linnaeus, 1758)	Hippoboscidae	Birds	EF531172	EF531215	EF531121	EF531150
Basilia boardmani Rozeboom, 1934	Nycteribiidae	Bats	EF531163	EF531225	n.a.	n.a.
Dipseliopoda setosa Theodor, 1955	Nycteribiidae	Bats	EF531183	EF531224	EF531125	n.a.
Penicillidia fulvida (Bigot, 1885)	Nycteribiidae	Bats	EF531174	EF531222	EF531124	EF531157
Brachytarsina speiseri (Jobling, 1934)	Streblidae	Bats	EF531175	n.a.	n.a.	n.a.
Megistopoda aranea (Coquillett, 1899)	Streblidae	Bats	EF531161	EF531219	n.a.	EF531154
Paratrichobius longicrus (Ribeiro, 1907)	Streblidae	Bats	EF531160	EF531217	n.a.	EF531152
Trichobius joblingi Wenzel, 1966	Streblidae	Bats	EF531191	EF531218	n.a.	EF531153

Table 2
Primer sequences used for PCR amplifcation and sequencing ([1]primer for initial amplification; [2]primer for reamplification)

Gene	Primer	Sequence
CAD	787F	GGD GTN ACN ACN GCN TGY TTY GAR CC[1] GTN GTN AAR ATG CCN MGN TGG GA[2]
CAD	1124R	CAT NCG NGA RAA YTT RAA RCG ATT YTC[1] TTN GGN AGY TGN CCN CCC AT[2]
COIa	mtd4	TAC AAT TTA TCG CCT AAA CTT CAG CC
COIa	mtd9	CCC GGT AAA ATT AAA ATA TAA ACT TC
COIb	mtd8	CCA CAT TTA TTT TGA TTT TTT GG
COIb	mtd12	TCC AAT GCA CTA ATC TGC CAT ATTA
28s	rc28a	AGC GGA GGA AAA GAA AC
28s	28c	GCT ATC CTG AGG GAA ACT TCG G
28s	rc28b	CCC GTC TTG AAA CAC GGA CC
28s	28p	GGC TTA CGC CAA ACA CTT CTA CGC
28s	rc28c	CCG AAG TTT CCC TCA GGA TAG C
28s	28e	CCT TAT CCC GAA GTT ACG
16s	16sf	CGC CTG TTT AAC AAA AAC AT
16s	16sr	TGA ACT CAG ATC ATG TAA GAA A

2.2. Phylogenetic analysis

The parsimony analyses were performed with TNT (Goloboff et al., 2003) using traditional search, 1000 random addition replicates, and TBR branch swapping. Non-parametric bootstrap values (Felsenstein, 1985) were calculated with the following settings: traditional search, 20.000 bootstrap replicates with 100 random addition analyses per replicate. Partitioned Bremer Support values (Baker and DeSalle, 1997) were calculated for individual nodes using TreeRot v2b (Sorenson, 1999) and PAUP* 4.0b10 (Swofford, 2002). Due to problems with sequencing several genes for *Brachytarsina speiseri* (Jobling, 1934), the dataset was analyzed both with this species included and excluded. We also tested the competing topological hypotheses for hippoboscoid relationships from Fig. 1 through constrained tree searches and statistical testing (Templeton test: Templeton, 1983). We defined constraint trees in MacClade (Maddison and Maddison, 2001) and used these in PAUP* (Swofford, 2002) for finding most parsimonious solutions under the specified constraints. All outgroup taxa were included as an unresolved polytomy. We then used the implementation of the Templeton test in PAUP* (*z*-test) for determining whether the competing topologies can be rejected based on our data.

We also analyzed our data using Bayesian analysis as implemented in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). All Bayesian sequence analyses were initiated from random starting trees and utilized the GTR+I+G model which was favored by the Akaike Information Criterion (AIC) as implemented in MrModeltest version 2.2 (Nylander, 2004). We partitioned the data set by gene and ran it for 1,500,000 generations. A tree was sampled every 300 generations, resulting in 5000 trees of which 25% were discarded as burn-in. Three independently repeated analyses resulted in similar tree topologies and comparable clade probabilities and substitution model parameters.

Key events in the evolution of Hippoboscoidea were reconstructed by mapping the following characters onto the most parsimonious tree: (1) hematophagy (presence/absence), (2) host choice (mammals/birds), (3) host specialization on bats (presence/absence), (4) adenotrophic viviparity (presence/absence), (5) motility of larva (capable of burrowing/incapable of burrowing/pupariation within female), (6) shedding of wings after finding host (presence/absence), (7) reduction of forewing size (presence/absence), (8) complete loss of forewing (presence/absence). These characters were mapped onto the most parsimonious tree excluding *Brachytarsina speiseri* using MacClade 4.03 (Maddison and Maddison, 2001) and ACCTRAN. The only multistate character was coded as non-additive.

3. Results

The length of the aligned sequences is 760 bp for CAD, 2561 bp for 28S rDNA, 542 bp for 16S, and 1264 bp for COI. In CAD, we found three trinucleotide insertions. One is shared between *Trichobius joblingi* Wenzel, 1966 and *Paratrichobius longicrus* (Ribeiro, 1907), whereas the other two are unique to *Megistopoda aranea* (Coquillett, 1899) and *Ornithomya avicularia* (Linnaeus, 1758), respectively. One nine-nucleotide indel is unique to *Drosophila melanogaster*. The total dataset is 5127 bp in length of which 1837 sites are parsimony informative.

The analysis excluding *Brachytarsina speiseri*, resulted in a single parsimonious tree with a total length of 8884 steps, a retention index (RI) of 0.441 and a consistency index (CI) of 0.6 (Fig. 2a). The interfamiliar relationships on this tree are identical to the proposal by Hennig (1973; Fig. 1). The monophyly of the Hippoboscoidea and its placement as sister group to the rest of the Calyptratae is strongly supported (BP = 90). The Glossinidae (BP = 100), the Hippoboscidae (BP = 94) and the bat flies (BP = 99) are all found to be monophyletic. Within the Glossinidae, two of the three currently recognized species groups (*palpalis*- and *morsitans*-species groups) are recovered as monophyletic with strong support (BP = 100 and 98, respectively). *Glossina brevipalpis* Newstead, 1910 of the *fusca* species group emerges as sister group to all remaining Glossinidae.

Within the Hippoboscoidea, the Glossinidae are the sister group of the remaining families (BP = 95). Within the Hippoboscidae, the Hippoboscinae (BP = 100) and Lipopteninae (BP = 100) are supported as monophyletic. The Ornithomyinae is paraphyletic. However, both constituent tribes, the Ornithomyini (BP = 100) and the Olfersiini (BP = 86) are monophyletic. At the genus level, *Ornithomya* and *Lipoptena* are both paraphyletic. The Nycteribiidae (BP = 100) and Streblidae (BP = 100) are both well supported monophyletic lineages, and together form a clade (BP = 99). The nycteribiid subfamily Nycteribiinae is also monophyletic (BP = 88).

The maximum parsimony analysis including *Brachytarsina speiseri* (Fig. 2b) yields a single most parsimonious tree with a tree length of 9008 steps, a consistency index (CI) of

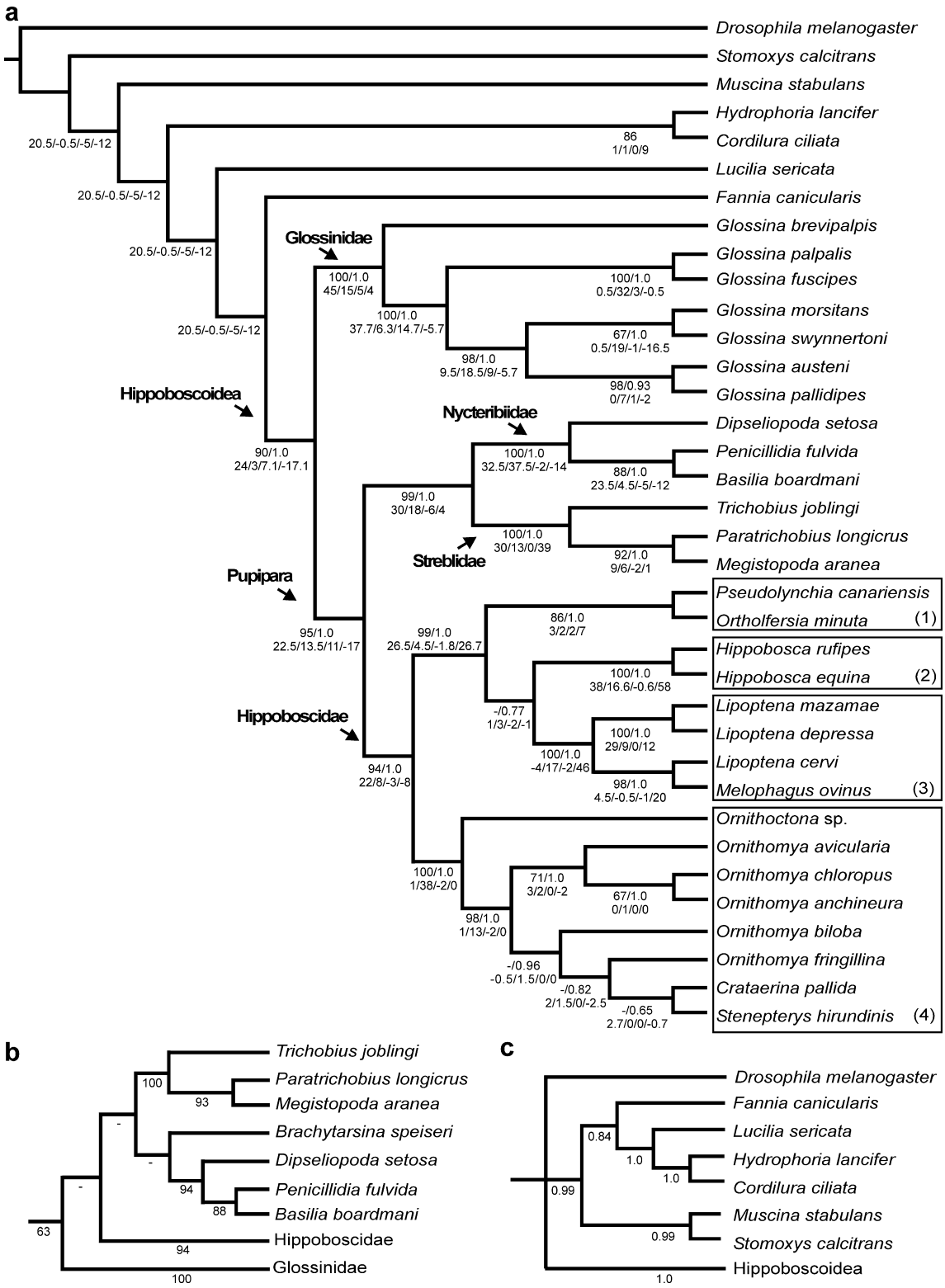

Fig. 2. (a) Maximum parsimony tree based on combined sequence data from CAD, COI, 16s, and 28s (numbers in first line are bootstrap support values and posterior probabilities; – = bootstrap support <50; numbers in second line are PBS values for CAD/COI/16s/28s; 1 = Olfersini; 2 = Hippoboscinae; 3 = Lipopteninae; 4 = Ornithomyini). (b) Subtree illustrating the topological difference between most parsimonious tree s with and without *Brachytarsina speiseri* (numbers = bootstrap support). (c) Subtree illustrating the topological difference between the most parsimonious tree in (a) and the Bayesian tree (numbers = posterior probabilities).

0.436, and a retention index (RI) of 0.569. The topology is nearly identical to that found in the analysis excluding *Brachytarsina speiseri* with the exception that within the bat fly clade *Brachytarsina speiseri* is placed as sister group to the Nycteribiidae, thus rendering the Streblidae paraphyletic, albeit without bootstrap support (Fig. 2b). The

Table 3
Testing competing phylogenetic hypotheses for Hippoboscoidea using the Templeton test (*z*-test; *difference between topologies is significant)

Hypothesis	Incl. *Brachytarsina speiseri*		Excl. *Brachytarsina speiseri*	
	Length diff.	Templeton (*z*-test, *p*-values)	Length diff.	Templeton (*z*-test, *p*-values)
McAlpine (1989)	54	0.017*, 0.017*, 0.012*, 0.012*	48	0.030*, 0.026*, 0.022*, 0.019*
Hennig (1973)	3	0.5775	0	1.0
Nirmala et al. (2001)	92	3 trees: <0.0001*	92	3 trees: <0.0001*
Dittmar et al. (2006) (ML)	47	0.013*, 0.020*, 0.018*	49	<0.0001*
Dittmar et al., 2006 (Bayesian)	48	0.022*, 0.020*, 0.031*, 0.027*	48	0.030*, 0.026*, 0.022*, 0.019*
Dittmar et al., 2006 (Parsimony)	79	0.0001*, 0.0006* 0.0004*, 0.0001* 0.0001*	81	0.0004*, 0.0003*

Bayesian analysis excluding *Brachytarsina speiseri* resulted in a tree with an identical ingroup topology to the most parsimonious tree depicted in Fig. 2a. Bayesian and parsimony analyses differed only in the resolution of outgroup relationships (Fig. 2c).

Hennig's hypothesis for the interfamiliar relationships of Hippoboscoidea is identical to the most parsimonious tree excluding *Brachytarsina*. However, when *Brachytarsina* is included, the Streblidae are paraphyletic, which disagrees with a statement by Hennig (1973) on the intrafamiliar relationships of the family that implies that he considered it monophyletic. Making Streblidae monophyletic requires three additional steps and the two topologies are not significantly different as judged by the Templeton test (Table 3). All topologies obtained by using previous hypotheses as topological search constraints (Fig. 1) require a large number of extra steps. These topologies are also rejected by the Templeton tests (Table 3). However, these test results have to be interpreted with care given that one of the test assumptions is that all sites evolve independently.

All life history characters had only one most parsimonious optimization. According to our most parsimonious tree, hematophagy evolved once and the hippoboscoid ancestor fed on mammal blood. Feeding on bats evolved once and feeding on birds twice. Adenotrophic viviparity involving the deposition of a single 3rd instar larva or puparium evolved in the hippoboscoid ancestor and the most parsimonious tree is compatible with the scenario that the deposited larva was initially motile (e.g., Glossinidae), then lost its ability to burrow in the soil (Hippoboscoidea), before starting to pupate already within the female (bat flies). Shedding of wings after finding a host evolved once and one species in this clade subsequently completely lost the forewings. Forewing loss is also observed in a second clade. Stenoptery (reduction of wing size) evolved twice.

4. Discussion

The Hippoboscoidea include some of the most important and interesting flies from a medical, veterinary, and morphological point of view. Yet, many questions regarding the evolution of these flies remain unanswered because key issues in Hippoboscoidea phylogenetics remained unresolved. One of the main obstacles with regard to trees based on morphology has been the large number of reductions due to extreme morphological adaptations associated with ectoparasitism. Determining primary homology was especially difficult for the highly modified wings, head, thorax, and legs that are commonly used in higher-level fly phylogenetics. Here, DNA sequences provide a particularly valuable source of phylogenetic information because molecular evolution in the standard genes used as phylogenetic markers is likely to be largely independent of the selection causing morphological adaptations to ectoparasitism. It is all the more surprising that the first two attempts at addressing interfamilial relationships in Hippoboscoidea using DNA sequences yielded conflicting results (Fig. 1 Nirmala et al., 2001; Dittmar et al., 2006). Nirmala et al. (2001) did not recover either of the two topologies that had been proposed based on morphology. Instead they found weak support for a third indicating a sister group relationship between the Hippoboscidae and the Nycteribiidae and a monophyletic group consisting of the Glossinidae+Streblidae. Conversely, Dittmar et al. (2006) found some support for McAlpine's (1989) hypothesis in parsimony and Bayesian trees by recovering Glossinidae+Hippoboscidae, but the placement of this clade relative to the bat flies varied and depended on the analysis method.

Our newly collected data appears to have strong phylogenetic structure, as indicated by high levels of branch support. These new data are also decisive in that alternative topologies can be rejected based on Templeton tests (Table 3). We are able to confirm the monophyly of the following key clades with high bootstrap support (BS): Hippoboscoidea (BS: 90), Glossinidae (BS: 100), Nycteribiidae (BS: 100), and Hippoboscidae (BS: 94). We can also corroborate Hennig's (1973) hypothesis of a sister group relationship between Glossinidae and all remaining Hippoboscoidea (''Pupipara'' BS: 95), and of a monophyletic bat fly clade consisting of Nycteribiidae and Streblidae (BS: 99).

We believe that two factors are likely responsible for finding strong support for interfamiliar relationships in our analysis. The first is better taxonomic coverage for the Hippoboscidae and Glossinidae. Nirmala et al. (2001) used three hippoboscoid species in their attempt to resolve

relationships within the Calyptratae, while Dittmar et al. (2006) concentrated on resolving the relationships within the bat fly families. For this reason, they included only a single glossinid and few hippoboscid species. Taxon sampling can have a major effect on clade recovery and support in phylogenetic inference (e.g., Hillis, 1998) especially in groups as diverse and old as holometabolous insects or calyptrate flies. Our more thorough taxon sampling was designed to test the monophyly of major hippoboscoid clades through more accurate optimization of ancestral states. This is especially important in cases where individual taxa may be highly autapomorphic in morphology or evolutionary rates. The second factor is the choice of genes. We used four genes and carried out a partitioned Bremer support (PBS) analysis in order to investigate their relative contributions to node support. As a summary of gene contribution to the total tree support, we calculated the sum of all PBS values (Σ_{PBS}) for each gene. Ranked in descending order of performance (e.g., overall influence on the parsimony tree topology), our genes were CAD ($\Sigma_{PBS} = 473.9$), COI ($\Sigma_{PBS} = 306.9$), 28S rDNA ($\Sigma_{PBS} = 64.75$), and 16S rDNA ($\Sigma_{PBS} = 2.45$). Only the worst-performing gene, 16S rDNA, had been used in previous attempts to resolve the phylogenetic relationships within the Hippoboscoidea (Nirmala et al., 2001; Dittmar et al., 2006). Overall, we find that in our analysis the nuclear genes CAD and 28S rDNA provide most of the support at the "basal" nodes while COI provides support at the tips.

It is noteworthy, however, that for some crucial nodes there is strong disagreement between the two nuclear genes. For example, the monophyly of the Hippoboscoidea and the monophyly of the Hippoboscidae are strongly contradicted by 28S rDNA, and CAD supports an arrangement of outgroup taxa that is in strong disagreement with all previous hypotheses of calyptrate relationships. Nonetheless, the additional evidence provided by just the small segment of CAD used here suggests that additional CAD sequence from contiguous regions of the gene may be quite valuable for additional studies of hippoboscoid relationships. In contrast, the small fragment of mitochondrial 16S rDNA contributes little or no information to our phylogenetic trees; it appears to us that this gene could be omitted from future studies of these taxa.

4.1. Classificatory implications

The monophyly of the Hippoboscoidea as a whole is supported by numerous morphological characters (Hennig, 1973; McAlpine, 1989) and has been confirmed in all previous molecular analyses (Nirmala et al., 2001; Dittmar et al., 2006) including the present study. It now seems established beyond doubt. However, the exact placement of the superfamily within the Calyptratae had not been firmly settled. In our analysis, the Hippoboscoidea are placed deeply nested within the Calyptratae (Fig. 2a), which is incongruent with McAlpine's (1989) hypothesis of a sister group relationship between Hippoboscoidea and the remaining calyptrate flies. However, our outgroup tree topology remains only poorly supported and the Bayesian analysis finds a similar placement of Hippoboscoidea as found by Nirmala et al. (2001) and Dittmar et al. (2006) in their parsimony and Bayesian analyses.

The monophyly of the Hippoboscidae has traditionally not been questioned and is here recovered with strong support (BP = 94). But our taxon sample for Hippoboscidae and Glossinidae also allows us to address classification issues within both families. Within the Hippoboscidae, our results are largely congruent with the family-level classification proposed by Maa (1969). The mammal parasites in the Lipopteninae and Hippoboscinae are sister groups and both monophyletic. However, Maa's (1969) Ornithomyinae consisting of Ornithomyini and Olfersiini is more problematic. The tribes are monophyletic and well supported, but the subfamily as a whole is paraphyletic with the Olfersiini being strongly supported (BS: 99) as sister group to Hippoboscinae+Lipopteninae. Within the Ornithomyini, our finding of paraphyly for the genus *Ornithomya* is not unexpected as the genera *Crataerina* and *Stenepteryx* were described by Maa (1963: 96) as "... a highly specialized form of the polyxenous, widely distributed genus *Ornithomya*", a view shared by Bequaert (1954: 69), who stated that "[t]he evolution of *Crataerina* from an *Ornithomya*-like(...) bird-fly is obvious". All three genera should be synonymized under *Ornithomya*. Synonymization could also repair the paraphyly of *Lipoptena* caused by the sheep ked, *Melophagus ovinus* (Linnaeus, 1758) which is deeply nested within *Lipoptena*. However, we suspect that there would be considerable resistance to a name change because both genus names are well established and *Melophagus*, which is intimately associated with the sheep ked, has priority over *Lipoptena*.

In the two previous molecular analyses of Hippoboscoidea, the Glossinidae had only been represented by a single taxon. For this reason, the authors were unable to test the monophyly of the glossinids and their subgenera. With our larger sample of species, we find the Glossinidae to be monophyletic (BS = 100) as was suggested by Hennig (1973), Griffiths (1972), and McAlpine (1989) based on morphology. In addition, we find support for the species group subdivision originally proposed by Newstead (1911) and later confirmed by studies on habitat preference (Jordan, 1974). The *fusca* species group is sister to the remaining Glossinidae as had been suggested by Newstead et al. (1924) and Bursell (1958). Our results therefore support these two proposed classifications over the alternatives proposed by Machado (1959) and Pollock (1973).

Relationships within the bat fly clade have been particularly difficult to resolve. Nirmala et al. (2001) did not recover it as monophyletic, while Dittmar et al. (2006) presented various results depending on which analytical method was used (see Fig. 1). Here, we find strong molecular support for the clade Streblidae+Nycteribiidae, a clade that had previously been suggested by numerous

authors familiar with the morphology of these families (e.g., Hennig, 1973; McAlpine, 1989). As in other analyses for this clade based on morphological or molecular characters, the monophyly of Nycteribiidae is unproblematic and the main problem is the delimitation and monophyly of the Streblidae. Dittmar et al. (2006) demonstrated streblid paraphyly by showing that Old World Streblidae+Nycteribiidae form a monophyletic group regardless of which analysis technique they used to evaluate their data. Our analyses corroborate the latter view, although weakly, because our matrix contains only a single Old World streblid for which we were unable to collect the full sequence data (Fig. 2). Dittmar et al. (2006) speculated that the difficulties in getting a consistent resolution of the basal relationships in the bat flies are the result of early rapid radiation within those groups. However, a visual inspection of branch lengths within the bat fly clade for our full combined molecular dataset did not reveal noticeably shorter branches than those found within the Hippoboscidae, and thus we see little evidence within our taxon and gene sample for a unique and rapid diversification in bat flies.

4.2. Host-shifts and diversification in Hippoboscoidea

Evolutionary changes in host use for organisms with specialized feeding habits such as these obligate vertebrate ectoparasites can be driven by reciprocal co-evolution between host and parasite (parallel cladogenesis), host tracking—in which parasites colonize and become established on closely related hosts—or by less predictable host shifts that are unconstrained by host taxonomy (Page, 2003; Labandeira, 2002). It is compelling to consider the potential for parallel cladogenesis in Hippoboscoidea. However, the age of the split between mammals and birds (Blair and Hedges, 2005: >310 MYA) greatly predates the earliest divergences found in extant hippoboscoid flies given that the earliest confirmed calyptrate fossil is only from the Oligocene (Michelsen, 2000). Parallel cladogenesis may be found in specific groups of bat flies, but definitive tests await more detailed and thoroughly sampled phylogenetic studies of nycteribiids and streblids.

Strikingly, within Hippoboscoidea true ectoparasitism originated only once in the common ancestor of the Pupipara (Fig. 3). This specialization involved a change from a

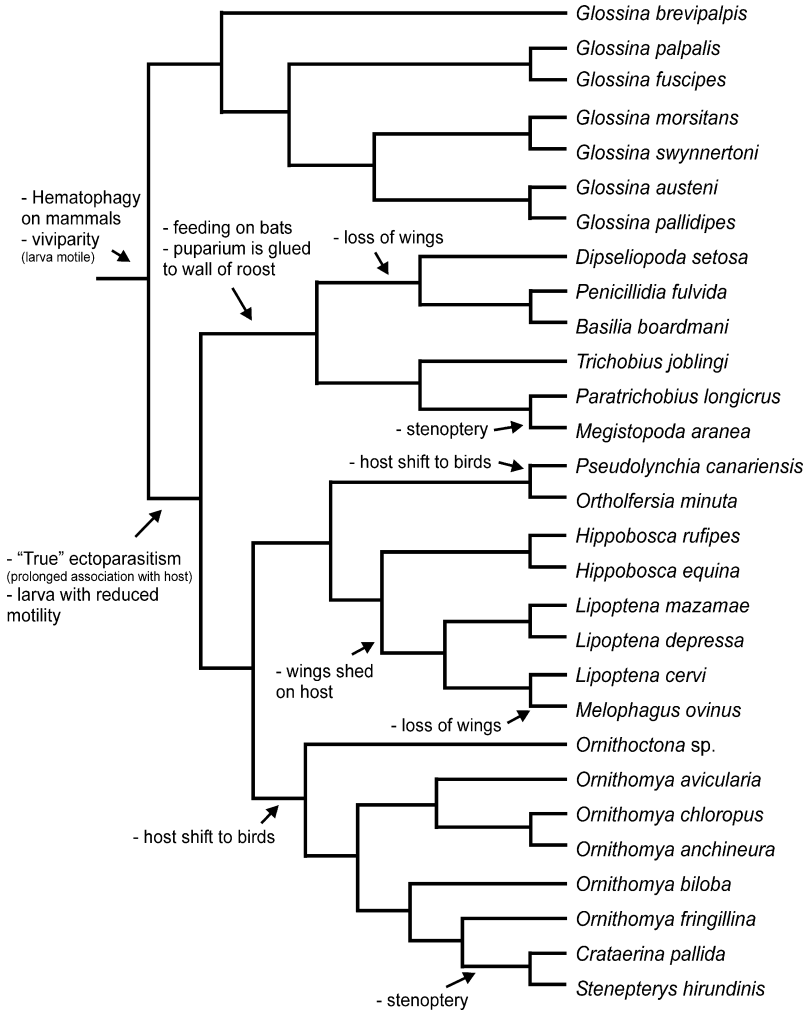

Fig. 3. Key events in the evolution of the Hippoboscoidea.

free-living, and blood-feeding fly (e.g., Glossinidae) to a fly with an obligate and close association with a particular vertebrate host. This specialization could have contributed to the large observed difference in species diversity between the sister groups Glossinidae (22 spp.) and Pupipara (630 spp.) given that it has been postulated that specialization of feeding structures, host finding behavior, and population subdivision associated with parasitism can spur species diversification (e.g., Mitter et al., 1988; Price, 1980, but see Wiegmann et al., 1993). Calyptratae includes multiple diverse lineages of flies with specialized feeding habits such as blood-feeding, parasitoidism, vertebrate endoparasitism and phytophagy. Better resolution of the phylogeny of Calyptratae, including a more exact placement for the Hippoboscoidea, will be necessary to fully gauge the possible affects of trophic specialization on diversification rates within this group.

When host-association is mapped onto our molecular phylogeny (Fig. 3), we find that mammal feeding is ancestral for the Hippoboscoidea. Based on this tree, feeding on birds has evolved at least twice; once in the Ornithomyini and once within the Olfersini while the ancestor of the Hippoboscidae was still feeding on the blood of mammals (contra Bequaert, 1954). However, this scenario is likely an oversimplification because our taxon sample is not extensive enough to illustrate the complete story. For example, we lack data for the bird-feeding *Struthiobosca* which is currently placed in the otherwise fully mammal-feeding Hippoboscinae. Better sampling of hippoboscines would also be necessary to allow determination of whether the ancestral host of this subfamily was a bird or mammal.

A major success story in Hippoboscoidea evolution was the host shift to bats. When we optimize host-association as a two-state character onto our tree (mammal vs. bird), we find that the shift to bats as hosts originated from species already feeding on mammals. The bat flies comprise approximately 500 species, a much larger number of species than their sister group, the Hippoboscidae with its approximately 150 species. Given that sister groups are of equal age, either the speciation rates in the former must have been higher or hippoboscid species experienced higher extinction rates. Our species sample is insufficient for studying the evolution of host specificity in Nycteribiidae and Streblidae, but more research in this area will be of central importance in understanding the major differences in species richness between the bat and hippoboscid flies.

4.3. Morphological and life history evolution in Hippoboscoidea

A partial or complete reduction of wings is often encountered within the Hippoboscoidea, and the tree topology in our analysis clearly indicates at least four complete or partial losses. Additional losses and cases of wing shedding will very likely be found once the taxon sampling is better for Streblidae. For example, *Ascodipteron* also lacks wings, but is not included in the data set. Within our taxon sample, the first reduction is within the Hippoboscidae where the monophyletic clade consisting of *Stenepteryx*+*Crataerina* is characterized by wings of reduced size. Indeed this is one of the reasons for the separation of these genera from the more ''generalized'' species in the genus *Ornithomya* with their fully developed wings. Secondly, in the subfamily Lipopteninae the genus *Melophagus* is characterized by the complete loss of wings and halteres. The other species in the Lipopteninae emerge from the puparium with fully developed wings and use flight to find an appropriate host. They then shed their wings close to the base and spend the remainder of their life on the host. The third reduction of wings is found in the Nycteribiidae, which, however, retain their halteres. Finally, the streblid *Megistopoda aranea* has straplike wings (i.e., is stenopterous), although, according to our cladogram, its ancestor was fully winged.

Loss of wings presumably allows easier and unhindered movement on the host and is common among ectoparasitic insects (Andersen, 1997). With at least four independent losses of wings within the superfamily, the Hippoboscoidea is no exception to this general pattern. However, while beneficial to an ectoparasite that needs to stay firmly attached while feeding and/or move about quickly on the host, the loss or reduction of wings must also severely impede dispersal and host finding. This problem may explain why wing loss is common in bat flies (>50% of all species). Bats are often gregarious and continue to use the same roosts for many generations which makes it relatively easy for bat flies to place their offspring in the direct vicinity of future hosts. A similarly conducive host ecology may also explain the complete loss of wings in the sheep ked, *Melophagus ovinus*. The females glue puparia to the fleece of the sheep and the frequent contact between host animals guarantees that the offspring will have access to a wide variety of hosts.

One of the most fascinating traits of all Hippoboscoidea is their ability to give birth to fully developed 3rd instar larvae. In the Glossinidae, the deposited larva is fully motile and actively burrows into the soil for pupariation. If the larva is deposited onto a suitable substrate, the larva can bury itself and start pupariation within 15 min. If deposited in an unsuitable location, it can delay the process by up to 6 h (Finlayson, 1967). In the Hippoboscidae, the deposited larvae are almost completely immobile, incapable of burrowing, and in most cases begin pupariation within the female (Bequaert, 1952). In the bat flies the immobilization is even more pronounced with deposited larvae being completely immobile. They are glued to the ceiling or wall of the bat roost by the female, after which they immediately pupariate (''pupiparity''). Our phylogenetic hypothesis is compatible with a gradual loss of motility. The formation of the puparium inside the female or immediately after deposition would be a synapomorphy for Pupipara, while gluing the puparium to the walls of bat roosts would be a synapomorphy of the Streblidae+Nycteribiidae.

5. Concluding remarks

Our sequence data provide convincing phylogenetic evidence for the monophyly of Hippoboscoidea, Glossinidae, Hippoboscidae, Nycteribiidae, Hippoboscinae, Lipopteninae, Ornithomyini, Olfersini, Nycteribiinae, Trichobiinae, the *Glossina palpalis*-, and the *Glossina morsitans* species groups within Glossinidae. The data also provide weak evidence for a paraphyletic Streblidae. Additional detailed studies based on a larger taxon sample within hippoboscoid families are needed to more fully investigate the evolution of specialization and host use in the families of Hippoboscidae as well as its specific phylogenetic position within the calyptrate Diptera. Nucleotide data should continue to provide an important independent phylogenetic data source for evolutionary investigations of these uniquely specialized flies.

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