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Phylogenetic analyses of mitochondrial and nuclear data in haematophagous flies support the paraphyly of the genus *Stomoxys* (Diptera: Muscidae).

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Short title: Phylogeny of *Stomoxys* flies

Abstract

The genus *Stomoxys* Geoffroy (Diptera; Muscidae) contains species of parasitic flies that are of medical and economic importance. We conducted a phylogenetic analysis including ten representative species of the genus including multiple exemplars, together with the closely related genera *Prostomoxys* Zumpt, *Haematobosca* Bezzi, and *Haematobia* Lepeletier & Serville. Phylogenetic relationships were inferred using Maximum Likelihood and Bayesian methods from DNA fragments from the cytochrome c oxidase subunit I (COI, 753 bp) and cytochrome b (CytB, 587 bp) mitochondrial genes, and the nuclear ribosomal internal transcribed spacer 2 (ITS2, 426 bp). The combination of mitochondrial and nuclear data strongly supports the paraphyly of the genus *Stomoxys* because of the inclusion of *Prostomoxys saegerae* Zumpt. This unexpected result suggests that *Prostomoxys* should be renamed into *Stomoxys*. Also, the deep molecular divergence observed between the subspecies *Stomoxys niger niger* Macquart and *S. niger bilineatus* Grünbreg led us to propose that they should rather be considered as distinct species, in agreement with ecological data. Bayesian phylogenetic analyses support three distinct lineages within the genus *Stomoxys* with a strong biogeographical component. The first lineage consists solely of the divergent Asian species *S. indicus* Picard which appears as the sister-group to all remaining *Stomoxys* species. The second clade groups the strictly African species *S. inornatus* Grünbreg, *S. transvittatus* Villeneuve*, S. omega* Newstead*,* and *S. pallidus* Roubaud. Finally, the third clade includes both African occurring and more widespread species such as the livestock pest *S. calcitrans* Linnaeus. Divergence time estimates indicate that the genus *Stomoxys* originated in the late Oligocene around 30 million years ago, with the major lineages diversifying in the Early Miocene between 20 and 15 million years ago at a time when temperate forests developed in the Northern Hemisphere.

1. Introduction

Stomoxys flies are principally haematophagous, and are associated with livestock and wildlife throughout the world. The most studied species, *Stomoxys calcitrans*, is an economically important pest of cattle and several studies have attempted to estimate its impact on cattle production (Miller *et al.*, 1973; Campbell *et al*., 1977; 2001). These flies represent a serious nuisance not only because of their painful bites and blood predation, but also because they are involved in the mechanical transmission of several pathogens, such as the *Capripoxvirus* causing a cattle disease (Lumpy skin disease), *Anaplasma marginale* the causative agent of severe bovine anaplosmosis, and *Dermatophilus congolensis* the causative agent of dermotophilosis (Zumpt, 1973; D'Amico *et al.*, 1996; Foil & Gorham, 2000).

The genus *Stomoxys* belongs to the tribe Stomoxyini in the subfamily Muscinae (De Carvalho, 1989; Couri & De Carvalho, 2003). This subfamily is part of the large family Muscidae comprising about 4,500 described species classified in 180 genera (De Carvalho *et al.,* 2005). Within Muscidae, phylogenetic analyses have been conducted at several taxonomic levels to assess the relationships among constitutive species (Couri & Pont, 2000; Couri & De Carvalho 2003; Schuhli & De Carvalho 2005; De Carvalho & Pont 2006; Schuhli et al. 2007). Recently, Nihei & De Carvalho (2007) carried out a cladistic analysis to assess the monophyly of the Muscini tribe. However, since the monograph of Zumpt (1973) who proposed the monophyly of Stomoxyini on the basis of morphological characters, and despite the medical and economic importance of these parasitic flies, no phylogenetic analysis has been carried out to assess the relationships within Stomoxyini. The flies of this tribe are easily recognized by their typical piercing/sucking mouthparts which differentiate them from the common housefly (*Musca domestica*) and relatives from the Muscini tribe. The Stomoxyini tribe consists of 10 genera and about 39 species reviewed by Zumpt (1973), the most medically and economically important species are members of the genera *Haematobosca*, *Haematobia* and *Stomoxys*.

The genus *Stomoxys*, which originated from the Old World, includes 18 species (Zumpt, 1973). Among these species, only *S. calcitrans* has a worldwide distribution and is a synanthropic fly. All other species are exclusively tropical, twelve of which are located on the African continent, four on the Asian continent, and one species, *S. sitiens* Rondani, has been reported in both Africa and Asia (Zumpt, 1973) (Table 1).

In this study, we address the phylogenetic relationships of the *Stomoxys* genus using mitochondrial (COI, CytB) and nuclear ribosomal (ITS2) nucleotide sequences. Mitochondrial DNA (mtDNA) has been widely used in systematic and many universal PCR primers are available for genes like COI and CytB (Avise, 2004). The ITS2 region is easy to amplify and have been used in previous phylogenetic studies of Diptera (Hwang, 2007; Thanwisai *et al*.,

2006). This work explores congruence and information content within the different molecular datasets analysed using probabilistic methods of phylogenetic reconstruction. Phylogenetic results are discussed and taken into consideration to propose a taxonomic revision of the group. Also, we propose estimations of divergence times for major clades within Stomoxyini based on a relaxed molecular clock approach. This allowed discussing the evolutionary history of *Stomoxys* species in its biogeographical context.

2. Materials and methods

2.1. Taxon sampling

The material used in this study belongs to the Laboratoire de Zoogéographie of the Université Paul-Valéry in Montpellier (France), and to the Department of Entomology of the Natural History Museum in London (United-Kingdom). For *Stomoxys* we sampled 11 representative species or subspecies. As outgroups, we incorporated *Prostomoxys saegerae*, the unique representative species of *Prostomoxys*, and *Haematobosca croceicornis*, a newly described species from Gabon (Pont & Dsouli, 2008). For this study, we also included sequences of *Haematobia irritans* and *Musca domestica* (Muscidae), as well as species belonging to the more distantly related Drosophilidae, Syrphidae and Dolichopodidae families in order to provide calibration points for molecular dating analyses (Wiegmann *et al*., 2003). Geographical origins of studied specimens and sequences accession numbers are listed in Table 2. Specimens are available upon request from the corresponding author and are stored in the Laboratoire de Zoogéographie of the Université Paul-Valéry in Montpellier (France).

2.2. DNA extraction, *amplification and sequencing*

Genomic DNA was extracted using the DNAeasy tissue Kit (QIAGEN) to a final volume of 180 µl. Amplifications by PCR, using the specific primer pairs described in Table 3, led to amplicons of different lengths for the COI (753 bp), CytB (587 bp) and ITS2 (~ 430 bp) regions. All PCR amplifications were performed in a 30µl reaction volume containing, at final concentrations, 200 µM dNTPs (diNucleotide Tri Phosphate), 10X buffer, 25µM of each primers and 0.5µl of Taq polymerase (Eurogentec Red GoldStar®), and 3 µl of purified DNA. Thermal cycling conditions for PCR were as followed: initial denaturation at 94°C for 4 min., 35 cycles of denaturation at 94°C for 40 sec., annealing at 48-50°C for COI, 57-58°C for CytB, and 60-62°C for ITS2, and extension at 72°C for 1 min. A final elongation step at 72°C for 10 min completed the DNA amplification process. Ten specimens for each species were

sequenced using Sanger sequencing on an ABI 3730 automatic sequencer at the Centre National de Séquençage (Génoscope) in Evry (France).

2.3. Phylogenetic analyses

The nucleotide sequences from COI, CytB, and ITS2 were automatically aligned using the multiple alignment program ClustalW 1.4 (Thompson *et al.*, 1994) using default parameters. Multiple sequence alignments were then adjusted by visual inspection, taking the sequences of *S. calcitrans* available in GenBank as a reference for each gene portion. Alignments were cleaned from problematic alignment blocks using Gblocks 0.91 (Castresana, 2000) using the following parameters: Minimum number of sequences for a conserved position = 38; Minimum number of sequences for a flanking position = 38; Maximum number of contiguous nonconserved positions = 8; Minimum length of a block = 5; Allowed gap positions = with half.

Probabilistic analyses were carried out on each individual datasets (COI, CytB, and ITS2). Maximum likelihood (ML) reconstruction was conducted using PAUP* 4.0b10 (Swofford, 2002). The best-fitting models of sequence evolution for different partitions were determined based on the Akaike Information Criterion (AIC) as implemented in jModelTest (Posada, 2008) using PHYML (Guindon & Gascuel, 2003) for calculating likelihood scores. ML heuristic searches were conducted with PAUP* using Tree Bisection Reconnection (TBR) branch-swapping on a Neighbor-Joining (NJ) starting tree using the best-fitting model and associated parameters selected by jModelTest. ML bootstrap proportions were obtained by repeating the same ML heuristic search on 100 pseudo-replicated datasets in order to evaluate the confidence for each node of the tree topology.

Crossed statistical SH tests (Shimodaira & Hasegawa 1999) of congruence between the three genes were performed in PAUP* by testing the best ML topology obtained from each gene against the topology inferred from their concatenation. These tests were run using each individual gene dataset and the concatenated dataset (see Table 4 for details).

Bayesian phylogenetic inference was conducted using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Three a priori partition schemes were tested for analyzing the concatenation of the three genes (COI, CytB, and ITS2): one single partition, a 3 gene partition, and a 7 partition scheme distinguishing 6 partitions for each codon position of the two coding genes (COI and CytB) plus a single partition for ITS2. For each partition, we used the best-fitting model selected by jModelTest. Bayesian inference under each partition scheme was conducted with two independent runs of four incrementally-heated Metropolis Coupled Markov Chain Monte Carlo (MCMCMC) starting from a random tree. MCMCMC were run for 3,000,000 generations with trees and associated model parameters being sampled every 300 generations. The initial 2,000 trees in each run were discarded as burn-in

samples and the harmonic mean of the likelihood was calculated by combining the two independent runs. The harmonic means were then used to compute the Bayes factor for the three possible partition comparisons (no partition *versus* 3 partitions by gene, no partition *versus* 7 partitions by codon and gene, and 3 partitions by gene *versus* 7 partitions by codon and gene). The best partition strategy was then determined by the 2ln Bayes factor criterion as recommended by Brandley *et al.* (2005). The 50% majority-rule consensus tree was then computed from the 16,000 trees sampled in the two independent runs under the best model. Posterior probabilities greater than or equal to 95 % are generally regarded as strong support for a clade (Wilcox *et al.,* 2002), but the correspondence with bootstrap support values is not exact (Douady *et al.*, 2003).

2.4. Molecular datings

Bayesian estimation of divergence times under relaxed-clock models was conducted using the Multidivtime package (Thorne & Kishino, 2002). We used the previously estimated Bayesian topology as the best hypothesis for *Stomoxys* phylogeny onto which divergence dates were estimated. The inclusion of species belonging to Drosophilidae (*Drosophila virilis, D. melanogaster* and *D. yakuba*), Syrphidae (*Simosyrphus grandicornis, Cheilosia longula* and *C. naruska*) and Dolichopodidae (*Dolichopus longula* and *D. nubilus*), which represent other brachyceran groups, allowed calibrating the tree. Indeed, based on a previously established timescale for brachyceran flies from Bayesian analyses of 28S rRNA data (Wiegmann *et al*., 2003), our tree was calibrated by using (1) the occurrence of Schizophora 87 million years ago (Mya) as the a priori expected number of time units between tip and root, and (2) the estimated date of the Drosophila/Musca split between 48 and 51 Mya as the calibration constraint. This previous molecular estimate was used in the absence of any relevant fossil calibration for Stomoxyini.

First, the program Baseml of the PAML package version 4.2b (Yang, 2007) was used to estimate the nucleotide frequencies, transition/transversion ratio, and rate heterogeneity among sites for the concatenated dataset. These values parameterize the F84+G nucleotide substitution model. Second, we calculated the branch lengths of the constrained topology and the associated variance-covariance matrix under this model using with the program Estbranches. Finally, the program Multidivtime was used to run a Markov chain Monte Carlo (MCMC) for estimating mean posterior divergence times on nodes with associated standard deviations and 95% credibility intervals (95% CredI) from the variance–covariance matrix produced by Estbranches. The MCMC was sampled 10,000 times every 100 cycles after a burn-in stage of 100,000 cycles. The prior for the expected number of time units between tip and root was set at 87 Mya (SD = 43 Mya). The estimated branch lengths obtained by Estbranches were used to estimate the median amount of evolution between the root and all

the tips of the ingroup. Other priors for gamma distribution of the rate at root node (rtrate and rtratesd) and the Brownian motion constant describing the rate variation (brownmean and brownsd) were derived from the median branch length of the phylogram. The highest possible number of time units between tip and root (Bigtime) was set to 119 Mya which corresponds to the upper bound of the 95% CredI for Schizophora as estimated by Wiegmann *et al*. (2003). The age of the node Musca/Drosophila was constrained using an upper bound of 51 Mya and a lower bound of 48 Mya (Wiegmann *et al*., 2003).

3. Results

3.1. Sequence characteristics

Alignment statistics and models selected for phylogenetic analyses for each data partitions are summarized in Table 5. Alignment of the mitochondrial genes was straightforward, as no indels were introduced. The alignment of COI sequences resulted in 753 nucleotide sites of which 228 (30.3%) are variable, and 198 (26.3 %) are parsimony informative. jModelTest identified the GTR+G+I model as the best-fitting model for the COI gene (lnL= -3610.24) based on the AIC. The gamma distribution shape parameter (alpha) was estimated to 0.2 which reveals strong among-site rate heterogeneity in this barcoding gene. The CytB alignment contained 587 sites of which 204 (34.8%) are variable and 161 (27.4 %) are informative. jModelTest also identified the GTR+G+I model as the best-fitting nucleotide substitution model for the CytB gene (lnL= -3121.46) with an estimated alpha of 0.62. Finally, the initial alignment of ITS2 sequences totalized 426 sites including indels. 131 sites corresponding to ambiguously aligned hypervariable regions were excluded from subsequent analyses by applying Gblocks. The remaining 295 sites contain 168 (57 %) variable sites of which 147 (50 %) are parsimony informative which makes this marker the most variable of our study. jModelTest identified the HKY+G model as the best-fitting model for the ITS2 marker (lnL= -2454.70). The gamma distribution shape parameter alpha was estimated to 0.43 in this ribosomal internal transcribed spacer.

3.2. Phylogenetic relationships

We evaluated topological congruence among the individual genes by computing crossed SH tests in which the highest-likelihood topologies obtained with each individual datasets and their concatenation were compared against each other (Table 4). While there is apparent incongruence among the individual markers, none of the three individual datasets in fact significantly rejects the ML topology supported by their concatenation (see last line of Table 4). This indicates that combining the three individual gene datasets leads to a

phylogenetic estimate that is compatible with the signal contributed by each individual gene. We therefore decided to concatenate the three datasets in order to maximize the number of characters analysed and to enhance the phylogenetic signal as advocated by Delsuc *et al.* (2002).

The total number of nucleotide positions in the concatenation was 1635. Bayes factor comparisons showed that the best-fitting model for the whole concatenation was the use of a single GTR+G model. Indeed, the 2ln Bayes factor criterion (Brandley *et al.*, 2005) was always in favour of H0 in the three partition scheme comparisons (2lnBF < 0.03) indicating that it was not worth partitioning in our case. The phylogenies obtained from ML and Bayesian methods under a single GTR+G were identical, but the Bayesian analysis provided higher support values for most of the nodes (Fig. 1). Our results support the monophyly of *Haematobia* and *Haematobosca* (PP = 1.0; BP_{ML} = 71), and this clade appears as the sister-group to the remaining Stomoxyini. The genus *Stomoxys* is rendered paraphyletic with strong support (PP = 1.0; BP_{ML} = 90) due to the inclusion of *Prostomoxys saegerae* within it, as a sister-group to *S. varipes* (Fig. 1). Strong support is obtained (PP = 1.00; BP_{ML} = 97) for *S. indicus* as representing the sister-group to all remaining species within the *Stomoxys* group (Fig. 1). Three major clades can be distinguished according to the Bayesian consensus tree within Stomoxyini. The first clade (clade A) is represented by *S. indicus* alone which is an Asian species. The second clade (clade B) is well supported (PP = 0.99 and BP_{ML} = 63) and groups species from African origin (*S. pallidus*, *S. omega*, *S. transvittatus*, and *S. inornatus*). Finally, the third clade (clade C), only supported by the Bayesian analysis (PP = 0.92), regroups all the remaining species containing cosmopolitan, African, and Asian species. This clade can be further divided into three subclades. The first subclade associates the two subspecies *S. niger niger* and *S. niger bilineatus* (PP = 0.98), the second subclade associates *P. saegerae* and *S. varipes* (PP = 0.89), and the third subclade composed of *S. calcitrans*, *S. sitiens,* and *S. bengalensis* (PP = 1.0; BP_{ML} = 56) with *S. sitiens* and *S. bengalensis* being sister-groups (PP = 1.0; BP_{ML} = 91) (Fig. 1).

3.3. Estimation of Divergence times

The divergence time estimates are presented as a chronogram where branching nodes correspond to the mean age estimated from the posterior distribution and its associated 95% CredI (Fig. 2). The divergence time between the *Stomoxys* genus and its *Haematobia* + *Haematobosca* sister-clade is estimated around 30.8 Mya (95% CredI: 40.3-22.3). The age estimate for the early emergence of *S. indicus* (clade A) within the genus *Stomoxys* is estimated at about 27 Mya (36.6-18.9). The divergence between clade B and clade C occurred around 20.8 Mya (29.6-13.6). According to this inferred timescale, the major lineages within the genus *Stomoxys* were present by the late Oligocene, whilst the

greatest amount of cladogenesis occurred during the Early Miocene. The two subspecies of *S. niger* (*S. niger niger* and *S. niger bilineatus*) separated around 16.3 Mya (24.2-10.3), a divergence time almost exactly similar to the one inferred for the separation between the species *S. inornatus* and *S. transvittatus* estimated at 16.4 Mya (24.6-10.0). Finally, the divergence between *P. saegerae* and *S. varipes* is estimated to have occurred 14.2 Mya (21.6-8.5) concomitantly with the separation of *S. calcitrans* from its *S. bengalensis*/*S. sitiens* sister-clade at 14.1 Mya (21.5-8.5).

4. Discussion

4.1. Phylogenetic relationships and taxonomy of the Stomoxys *group*

The phylogenetic analyses performed in the present study allowed reconstructing a phylogenetic framework for the major constitutive species of the genus *Stomoxys* and closely related genera. Our analyses strongly support the monophyly of Stomoxyini including a monophyletic group consisting of *Haematobia* and *Haematobosca* as a sister-clade to all remaining Stomoxyini. However, according to our results the genus *Stomoxys* sensu stricto is found to be paraphyletic due to the unexpected position of *Prostomoxys saegerae* which appears to be well nested within the *Stomoxys* group as a sister-group to *S. varipes* (Fig. 1). Zumpt (1973) created the genus *Prostomoxys* for the sole species *P. saegerae*. According to Zumpt's identification key for Stomoxyini, *Prostomoxys* is characterized by maxillary palps that are as long as the proboscis, whereas *Stomoxys* is characterized by palps that are shorter than half the length of the proboscis. This key includes no other diagnostic character for the distinction of this genus from *Stomoxys*. Zumpt (1973) mentioned that the plesiomorphic form of the Stomoxyini mouthparts can be accepted as the maxillary palps being about as long as the proboscis. Based on our phylogenetic study there appears to be no reason to incorporate this species in a separate genus as *P. saegerae* is in fact closely related to *Stomoxys* species. *Prostomoxys* should thus be synonymised with *Stomoxys*, and the species *Prostomoxys saegerae* must be renamed into *Stomoxys saegerae*. The long palp characters would be better considered as mere specific traits, since they are likely to be plesiomorphic.

Within *Stomoxys* lato sensu (including *P. saegerae*), our phylogenetic analyses strongly support the placement of *S. indicus* as the sister-group to the remaining *Stomoxys* species. Taxonomically, *S. indicus* is considered to be highly variable, with a number of synonyms used in the entomological literature. As mentioned by Zumpt (1973): "it is possible that a numerical taxonomic study based on great numbers of specimens from various populations may reveal that several subspecies may be retained in the future". This species

is described as the most common *Stomoxys* species in the Oriental region after *S. calcitrans* and has been recorded from India to the Pacific islands of Fiji and Samoa, including China and Japan. Its geographic range also reaches the Palaearctic region on the occidental side. Moreover, it has also been recorded in a number of South-East Asian countries such as Thailand (Masmeatathip *et al*., 2006), Malaysia, Viet Nam, Taiwan, Borneo, Sumatra, and Java (Zumpt, 1973). Since both of our samples are from Thailand origin, they provide no information on the genetic diversity among populations of the species, but they reveal its evolutionary distinctiveness from other members of the genus *Stomoxys*.

The remaining *Stomoxys* species included in our study can be divided in two main clades. Clade B contains only African species and clade C groups both African and more geographically widespread species (Fig. 1). Clade B regroups *S. inornatus*, *S. transvitattus*, *S. omega* and *S. pallidus* sampled from different parts of the Ethiopian region. These species are ubiquitous and are restricted mainly to forest ecotones where they feed especially on wildlife fauna (Mavoungou *et al.*, 2008). This large clade appears to be divided in two main subclades associating *S. inornatus* and *S. transvittatus* on one side, and *S. omega* and *S. pallidus*, on the other side. *S. inornatus* and *S. transvittatus* have a diurnal activity whereas *S. omega* and *S. pallidus* have a crepuscular activity (Duvallet, unpublished data). These observations would fit well with the proposed phylogeny. However, the information content of ecological data for phylogeny is probably limited since activity levels in *Stomoxys* have been shown to be highly dependent upon geographical origin (primary forest, secondary forest and human-modified area) (Mavoungou *et al*., 2008), climate (temperature, humidity, and solar radiation level) (Kangwagye, 1974; Charlwood & Lopes, 1980), trapping method, and physiological state of individuals (Simmond, 1944; La Breque *et al*., 1975).

The third clade C can be divided into three subclades, two of them being exclusively African, and one being more geographically widespread. The first subclade groups *S. niger niger* and *S. niger bilineatus*, considered as two subspecies of *S. niger* according to Zumpt (1973), whereas the second subclade associates *P. saegerae* and *S. varipes*. Solving species boundaries between closely related species is notoriously difficult in these parasitic flies. Based on the tree topology, *S. niger niger* and *S. niger bilineatus* sequences form distinct phylogenetic clusters. The pairwise distance between *S. niger niger* and *S. niger bilineatus* based on COI sequences is 8.2%. The use of DNA sequences for species delimitation has been widely criticized (Tautz *et al*., 2002; DeSalle *et al*., 2005; Meier *et al*., 2006). The wide overlap between intraspecific and interspecific variability observed in Diptera COI sequences (0% to 15.5%) (Meier *et al*. 2006) is especially problematic here, since the pairwise distance for these subspecies falls into this overlapping area. However, the genetic distance between *S. niger niger* and *S. niger bilineatus* based on the nuclear ITS2 is also quite large with 14.3 %. Moreover, our molecular dating analysis estimates the

separation between the two subspecies around 16.3 Mya (Fig. 2). This is almost as old as the separation between *S. inornatus* and *S. transvittatus* (c.a. 16.4 Mya) and is comparable with the divergence observed between *D. melanogaster* and *D. yakuba* (c.a 16.2 Mya). It is also more ancient than the divergence between *S. omega* and *S. pallidus* (c.a. 12.4 Mya), and even more ancient than the split between *S. varipes* and *P. saegerae* (c.a. 14.2 Mya) (Fig. 2).

Morphologically*, S. niger niger* and *S. niger bilineatus* are distinguished only by the colour of their tibiae and tarsi*.* Mavoungou *et al*. (2008) described *S. niger bilineatus* and *S. niger niger* as sympatric species, the former being abundant in savannas with abundant wild fauna, while the latter is more associated with anthropized area. Mihok *et al.* (1996) also pointed out differences in habitat affinities, sex ratio and activity patterns between these species. Given morphological observations, ecological isolation and molecular data, it appears very likely that *S. niger niger* and *S. niger bilineatus* belong to different species. Consequently, we suggest raising them to full specific status and to use *S. niger* and *S. bilineatus* as species names.

Finally, the third subclade within clade C associates *S. calcitrans*, *S. sitiens* and *S. bengalensis* Picard. *S. bengalensis* occurs only in Asia, *S. sitiens* is found in both the African and Asian continents, while the livestock pest *S. calcitrans* has a worldwide distribution. *S. calcitrans* and *S. sitiens* appeared to be strictly human commensals since they have invariably been found in association with human activity, including inside buildings.

4.2. Molecular timescale and biogeography of the Stomoxys *group*

Our molecular estimate of the separation between Stomoxyini and Muscini falls into the Late Eocene epoch (c.a. 34.9 Mya) (Fig. 2). This divergence time is compatible with the record of the oldest Muscidae fossils in the Eocene and the Lower Miocene, 50-20 Mya (Evenhuis, 1994). The oldest Muscini fossil (about 20-15 Mya) was described from Dominican amber (Pont & De Carvalho, 1997), whereas no Stomoxyini fossil has been reported yet. The results presented here show that the radiation of the major Stomoxyini groups largely overlaps with the hypothetical time span (mid-Oligocene to mid-Miocene) during which temperate forests developed in the Northern Hemisphere (Guo *et al*., 2002).

The genus *Stomoxys* appears to diverge from other Stomoxyini genera in the Oligocene (c.a. 30 Mya). An Oriental origin of the *Stomoxys* genus lato sensu is tentatively suggested, owing to the strong support obtained for the basal branching of *S. indicus* (Clade A) and its absence from Africa. Under such a scenario, the common ancestor of the remaining *Stomoxys* members would have then split into two groups in the Early Miocene (c.a. 21 Mya), with one group being distributed in the Ethiopian region (Clade B), while the other colonized Asia (Clade C). The collision between Eurasia and the Arabo-African plate

was initiated by the Oligocene, but the Early Miocene period seems to have been key for the establishment of intercontinental pathways permitting faunal exchanges between the Afro-Arabian and Asia plates (Bernor *et al*., 1987). This biogeographical event fits well with our estimates of the split between clades B and C occurring within this period. Continental exchanges through migration events continued until the Late Miocene and could explained the occurrence of *S. calcitrans* and *S. sitiens* in both the African and Asian continents. Migration until the Late Miocene may have been facilitated by a forest connection that existed between the African and Oriental regions at this epoch (Moreau, 1963).

Our study is a first step into the molecular phylogenetic analysis of the *Stomoxys* genus. Yet, our results point to the need for a taxonomic revision of the Stomoxyini tribe. Since very few data on the biology and ecology of this group are currently available it would be necessary to advance the knowledge in these basic research areas. This is a prerequisite for better understanding how these species originated and diversified in a biogeographical context.

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Figure legends

Fig. 1. Phylogenetic relationships among 33 dipteran species inferred from the concatenation of *COI*, *CytB* and *ITS2* (1635 nucleotide sites). This phylogram is the 50% majority rule consensus tree obtained with Bayesian inference under the GTR+G model. Numbers at nodes indicate Posterior Probabilities (PP) / Maximum Likelihood Bootstrap Proportions (BP_{ML}). Black circles indicate nodes receiving maximum values of PP and BP and dashes mark nodes above the 50% level. The three main lineages identified within the *Stomoxys* group are labeled A, B, and C.

Fig. 2. Chronogram resulting from the relaxed molecular clock Bayesian analysis of the concatenation of *COI*, *CytB* and *ITS2* (1635 nucleotide sites). Mean divergence times are indicated at nodes and node bars represent 95% Credibility Intervals. Scale is in Millions of years before present. Species names belonging to the genus *Stomoxys* have been abbreviated (S.). The *Drosophila*/*Musca* calibration constraint is shown in black. Tertiary Epochs are indicated following the geologic timescale 2004 of the Geological Society of America (Gradstein *et al.*, 2004). Plio.: Pliocene; Pleist.: Pleistocene.

Table 1. *Stomoxys* species distributions as mentioned by Zumpt (1973).

Species	Distribution
S. bengalensis Picard	India to Java
S. boueti Roubaud	Benin (ex-Dahomey), Congo
S. calcitrans (Linnaeus)	Cosmopolitan
S. indicus Picard	Oriental region and neighbouring Paleartic territories
S. inornatus Grünberg	Tropical Africa
S. luteolus Villeneuve	Central and East Africa
S. niger Macquart	
S. niger niger	Ethiopian and Madagascan regions
S. niger bilineatus	Ethiopian and Madagascan regions
S. ochrosoma Speiser	Central and East Africa
S. omega Newstead	Ethiopian Region
S. pallidus Roubaud	Tropical Africa
S. pullus Austen	India
S. sitiens Rondani	Ethiopian and Oriental regions, in Egypt it reaches the Palearctic region
S. stigma Van Emden	Uganda – Congo
S. taeniatus Bigot	Ethiopian region
S. transvittatus Villeneuve	Southern and Central Africa
S. uruma Shinonaga & Kano	Oriental region
S. varipes Bezzi	East and Central Africa southward to Rhodesia
S. xanthomelas Roubaud	Congo – Tanzania – Uganda

Table 2. Species sampling, source localities, and GenBank accession numbers.

Species	Origin	COI	CytB	ITS2
Stomoxys calcitrans	India	EU836073-EU836074	EU851301	NA
	Thailand	EU836075-EU836082	EU851303-EU851308	EU851200-EU851208
	Cameroun	EU836070	EU851302	EU851199
Stomoxys indicus	Thailand	EU836083-EU836092	EU851309-EU851318	EU851209-EU851218
Stomoxys sitiens	Thailand	EU836131-EU836138	EU851357-EU851364	EU851251-EU851257
	Burkina Faso	FJ386382	FJ386381	NA
Stomoxys bengalensis	Thailand	EU836060-EU836068	EU851281-EU851288	EU851191-EU851198
Stomoxys niger niger	Gabon	EU836110-EU836115	EU851327-EU85133	EU851228-EU851237
Stomoxys niger bilineatus	Gabon	EU836101-EU836108	EU851289-EU851295	EU851219-EU851227
Stomoxys varipes	Ethiopia	EU836147-EU836148	EU851372-EU851374	EU851189-EU851190
Stomoxys transvittatus	Gabon	EU836139-EU836145	EU851365-EU851370	EU851258-EU851265
Stomoxys inornatus	Gabon	EU836093-EU836100	EU851319-EU851326	EU851179-EU851188
Stomoxys omega	Gabon	EU836116-EU836121	EU851339-EU851346	EU851238-EU851244
Stomoxys pallidus	Gabon	EU836122-EU836130	EU851347-EU851356	EU851245-EU851250
Prostomoxys saegerae	Gabon	EU836055-EU836059	EU851275-EU851280	EU851266-EU851270
Haematobosca croceicornis	Gabon	EU836053-EU836054	EU851273-EU851274	EU851271-EU851272
Haematobia irritans	-	AY526195*	DQ029097*	DQ437515*
Musca domestica	-	AF104622*	DQ657064*	Z28417*
Drosophila yakuba	-	NC_001322*	NC_001322*	Z28416*
Drosophila melanogaster	-	NC_001709*	NC_001709*	EU306667*
Drosophila virilis	-	DQ471577*	AY646771*	Z28415*
Cheilosia naruska	-	DQ417498*	NA	FJ028661*
Cheilosia longula	-	FJ158631*	NA	FJ158631*
Simosyrphus grandicornis	-	NC_008754*	NC_008754*	NA
Dolichopus nubilus	-	AY958244*	AY958244*	NA
Dolichopus excisus	-	AY958245*	AY958245*	NA

The sequences marked with an asterisk (*) were obtained from GenBank, (NA) no available data.

Table 3. COI, CytB and ITS2 Primers used for amplifications and sequencing.

Primers	Sequences (5'->3')	Reference
C1-J-2813 (direct)	CAACATTTATTTTGATTTTTTGG	Simon *et al*.,1994; 2006
TL2-N-3014 (reverse)	TCCATTGCACTAATCTGCCATATTA	
CB-J10933 (direct)	GTTTTACCTTGAGGACAAATATC	Simon *et al*., 1994
CB-N11526 (reverse)	TTCAACTGGTCGAGCTCCAATTCA	
ITS2A (direct)	TGTGAACTGCAGGACACAT	Sharpe *et al*., 2000
ITS2B (reverse)	TATGCTTAAATTCAGGGGGT	

Table 4. Statistical tests of topological congruence among the three genes using crossed SH tests (Shimodaira and Hasegawa 1999).

	Datasets			
Topologies	**CO1**	**CYTB**	**ITS2**	**Concatenation**
CO1	**5207.93**	42.75*	72.91*	70.32^{ns}
CYTB	145.87*	**4174.34**	144.34*	297.63*
ITS2	162.32*	200.42*	**2452.65**	323.39*
Concatenation	12.89^{ns}	23.69^{ns}	3.86^{ns}	**12265.83**

Log-likelihood values of ML topologies inferred from each individual gene (CO1, CYTB and ITS2) and from their concatenation were computed using each of the four data matrices and then compared with the corresponding highest log-likelihood value (in bold). The difference in log-likelihood values derived from these crossed comparisons are indicated. An asterisk (*) signifies that the tested topology is significantly worse at the 5% level than the best ML topology inferred from the corresponding dataset. ns means that this difference is not statistically significant.

Table 5. Best fitting models and associated maximum likelihood parameters obtained for the gene partitions used in this study.

Dataset	Sites	A	T	C	G	Best Model	Alpha	Pinv
COI	753	0.31	0.41	0.14	0.14	GTR+I+G	0.2	0.01
First Pos.	251	0.46	0.49	0.04	0.01	TrN+G	0.45	-
Second Pos.	251	0.29	0.32	0.12	0.27	GTR+G	0.16	-
Third Pos.	251	0.21	0.41	0.24	0.14	HKY+I	-	0.80
CytB	587	0.32	0.42	0.14	0.12	GTR+I+G	0.62	0.35
First Pos.	196	0.30	0.32	0.16	0.21	TIM+G	0.24	-
Second Pos.	196	0.20	0.46	0.29	0.11	HKY+I	-	0.68
Third Pos.	195	0.45	0.48	0.06	0.01	HKY+G	0.77	-
ITS2	295	0.36	0.37	0.10	0.17	HKY+G	0.43	-
Concatenation	1635	0.33	0.11	0.12	0.44	GTR+I+G	0.95	0.44

Figure 1

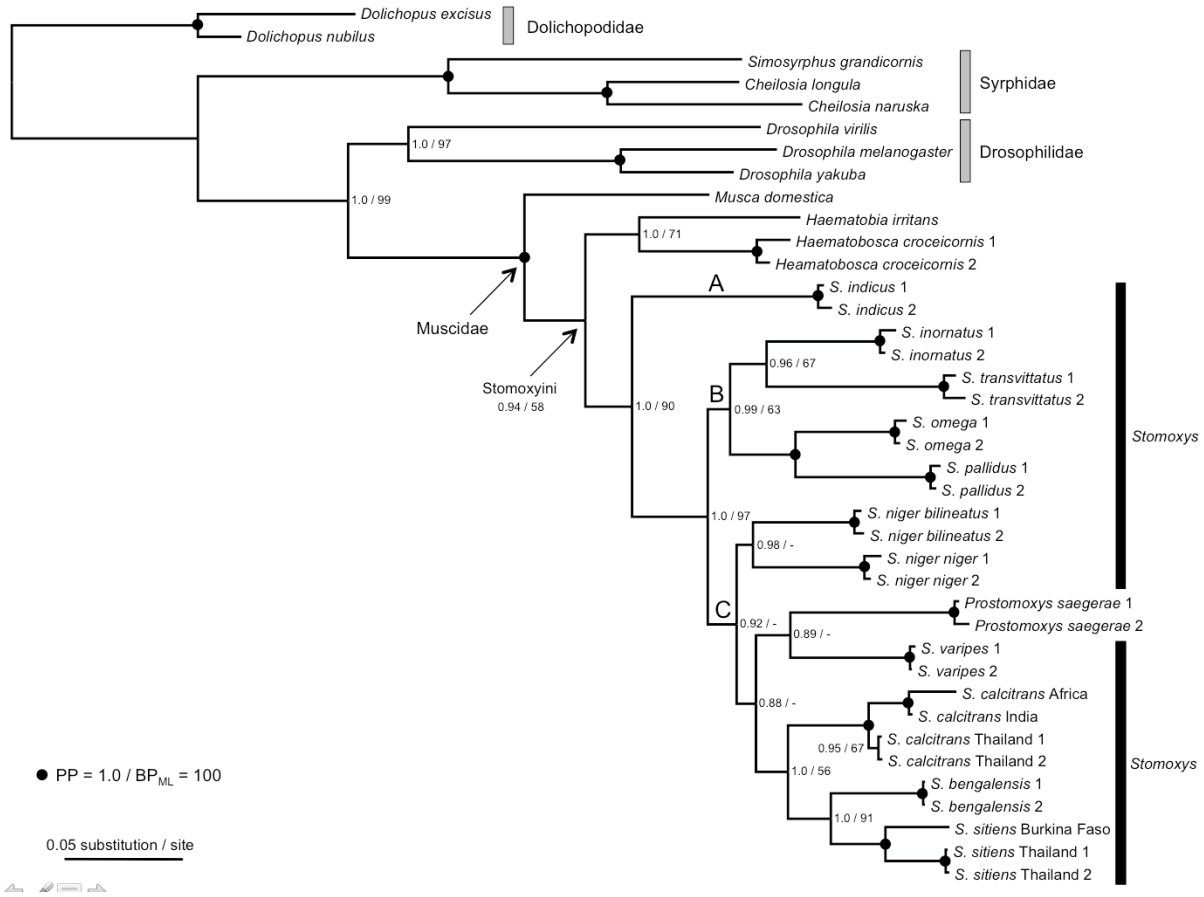
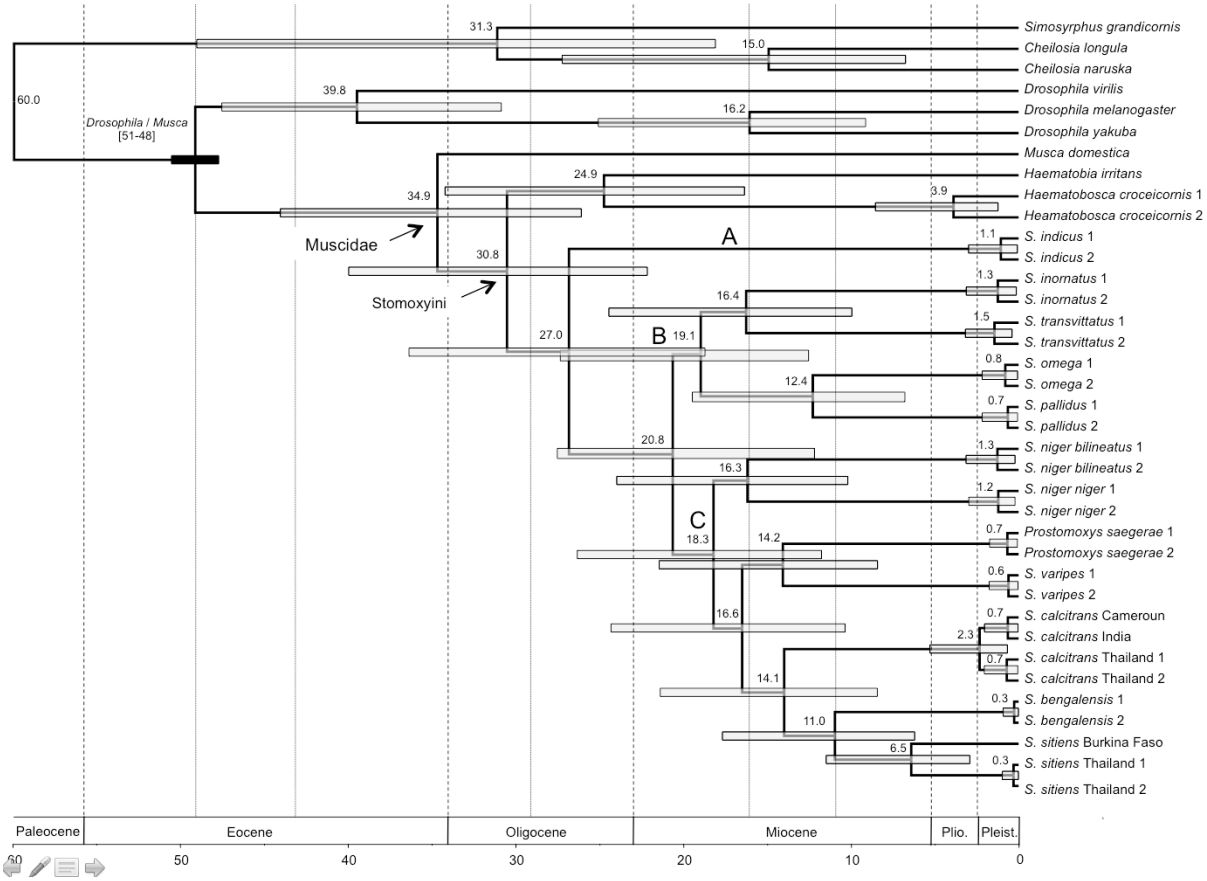

Dolichopus excisus
Dolichopus nubilus
Dolichopodidae
Simosyrphus grandicornis
Cheilosia longula
Cheilosia naruska
Syrphidae
Drosophila virilis
Drosophila melanogaster
Drosophila yakuba
Drosophilidae
1.0 / 97
1.0 / 99
Musca domestica
Haematobia irritans
1.0 / 71
Haematobosca croceicornis 1
Heamatobosca croceicornis 2
Muscidae
Stomoxyini
0.94 / 58
A
S. indicus 1
S. indicus 2
S. inornatus 1
S. inornatus 2
0.96 / 67
S. transvittatus 1
S. transvittatus 2
B
0.99 / 63
1.0 / 90
S. omega 1
S. omega 2
S. pallidus 1
S. pallidus 2
Stomoxys
1.0 / 97
S. niger bilineatus 1
S. niger bilineatus 2
0.98 / -
S. niger niger 1
S. niger niger 2
C
Prostomoxys saegerae 1
Prostomoxys saegerae 2
0.92 / -
0.89 / -
S. varipes 1
S. varipes 2
0.88 / -
S. calcitrans Africa
S. calcitrans India
0.95 / 67
S. calcitrans Thailand 1
S. calcitrans Thailand 2
1.0 / 56
S. bengalensis 1
S. bengalensis 2
1.0 / 91
S. sitiens Burkina Faso
S. sitiens Thailand 1
S. sitiens Thailand 2
Stomoxys
PP = 1.0 / BP_ML = 100
0.05 substitution / site

Figure 2


31.3
15.0
60.0
39.8
16.2
Drosophila / Musca
[51-48]
24.9
34.9
3.9
Muscidae
A
30.8
Stomoxyini
1.1
1.3
16.4
1.5
27.0
B 19.1
0.8
12.4
0.7
20.8
1.3
16.3
1.2
C
18.3
14.2
0.7
0.6
16.6
0.7
2.3
0.7
14.1
0.3
11.0
6.5
0.3
Simosyrphus grandicornis
Cheilosia longula
Cheilosia naruska
Drosophila virilis
Drosophila melanogaster
Drosophila yakuba
Musca domestica
Haematobia irritans
Haematobosca croceicornis 1
Heamatobosca croceicornis 2
S. indicus 1
S. indicus 2
S. inornatus 1
S. inornatus 2
S. transvittatus 1
S. transvittatus 2
S. omega 1
S. omega 2
S. pallidus 1
S. pallidus 2
S. niger bilineatus 1
S. niger bilineatus 2
S. niger niger 1
S. niger niger 2
Prostomoxys saegerae 1
Prostomoxys saegerae 2
S. varipes 1
S. varipes 2
S. calcitrans Cameroun
S. calcitrans India
S. calcitrans Thailand 1
S. calcitrans Thailand 2
S. bengalensis 1
S. bengalensis 2
S. sitiens Burkina Faso
S. sitiens Thailand 1
S. sitiens Thailand 2
Paleocene
Eocene
Oligocene
Miocene
Plio.
Pleist.
60
50
40
30
20
10
0