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Molecular phylogenetics of Oestroidea (Diptera: Calyptratae) with emphasis on Calliphoridae: Insights into the inter-familial relationships and additional evidence for paraphyly among blowflies

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ABSTRACT

The superfamily Oestroidea, comprising ~15,000 species, is a large and ecologically diverse clade within the order Diptera. Among its six commonly recognized families, Calliphoridae seems to be crucial for understanding evolutionary relationships in the group, as it is recognized as a controversial paraphyletic grouping. To further investigate this matter, the ITS2, 28S, COI and 16S regions were used to infer phylogenetic relationships in Oestroidea with maximum-parsimony (MP), maximum-likelihood (ML) and Bayesian inference (BI) methods. For the BI analyses, a deep evaluation of different data partitioning strategies was conducted, including consideration of structural conformation (ITS2 and 16S) and codon position (COI) information. Results suggest the existence of two main clades in Oestroidea: (Tachinidae + Mesembrinellinae) and (Rhiniinae, (Sarcophagidae + Calliphoridae *sensu stricto*)). Oestridae was recovered as sister group of the remaining Oestroidea in the MP trees while it was placed closer to the (Rhiniinae + Sarcophagidae + Calliphoridae *sensu stricto*) group in the ML and BI trees. A paraphyletic Calliphoridae was recovered, confirming the exclusion of Rhiniinae, a clade recently promoted to family status and therefore already excluded. Mesembrinellinae could also be considered a distinct group apart from Calliphoridae, although further studies are required. Consideration of structural and codon position information led to a significant increase in the log-likelihoods of the analyses, which were accompanied by small changes in the inferred topologies, branch lengths and posterior probability support values. However, as model complexity increases, so does uncertainty across the estimated parameters, including tree topologies, and phylogenies inferred under very parameter-rich models may be less reliable even when possessing higher log-likelihoods.

1. Introduction

The family Calliphoridae (Diptera: Calyptratae: Oestroidea), whose members are commonly known as blow flies, is a very diverse and heterogeneous group comprising approximately 1500 species in a worldwide distribution (Pape et al., 2011). The family is better known for its saprophagous and myiasis-causing members in the subfamilies Chrysomyinae, Calliphorinae and Luciliinae (de Azeredo-Espin and Lessinger, 2006; Stevens and Wallman, 2006; Stevens et al., 2006), which have synanthropic habitats and great importance in forensic, veterinary, medical and economic issues (Zumpt, 1965; Guimarães et al., 1983; Hall and Wall, 1995; Amendt et al., 2004). However, the group encompasses an even greater diversity of feeding habits and breeding environments, including hematophagous parasitism of birds and mammals (e.g. species of *Protocalliphora*, *Trypocalliphora* and *Auchmeromyia*), parasitism of terrestrial gastropods (e.g. species of *Melanomya*, *Melinda* and many species in the subfamily Amenniinae) and earthworms (e.g. species of *Bellardia* and *Pollenia*) and close associations with termites' and ants' nests (e.g., species of *Bengalia*, *Tricyclea*, *Hemigymnochaeta* and *Termitocalliphora* and some species of Rhiniinae).

Historically, Calliphoridae has been a controversial group concerning both its composition and its monophyletic status. Regarding family monophyly, Lehrer (1970), Rognes (1991), McAlpine (1989) and Pape (1992) provided corroborating evidence (although recognizing its fragility), but both Hennig (1973) and Griffiths (1982) have noted lack of support for it. More recently, Rognes (1997) and Kutty et al. (2010) have provided evidence for

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non-monophyly based on analyses of morphological and molecular characters, respectively.

The classification into subfamilies and tribes is also controversial and the number of subfamilies attributed to Calliphoridae is variable, ranging from two (Shewell, 1987: Calliphorinae, with the tribes Calliphorini, Polleniini, Angioneurini and Luciliini; and Chrysomyinae, with the tribes Chrysomyiini, Rhiniini and Phormiini) to 13 (Rognes, 1986, 1991, 1997: Chrysomyinae, Calliphorinae, Luciliinae, Toxotarsinae, Melanomyinae, Auchmeromyinae, Bengaliinae, Polleniinae, Mesembrinellinae, Phumosiinae, Rhiniinae, Helicoboscinae and Ameniinae), with some intermediate schemes (e.g., Hennig, 1973: Calliphorinae, Chrysomyinae, Mesembrinellinae, Ameniinae and Rhiniinae).

The controversially monophyletic status of Calliphoridae, with recent evidence of paraphyly, allied with a currently poor understanding of Oestroidea interfamilial relationships, implies the possibility that the family, as traditionally considered, could be further divided into smaller groups with variable and uncertain placements among the remaining oestroid families. This makes Calliphoridae the key family for understanding the evolution and phylogeny of the Oestroidea (McAlpine, 1989; Rognes, 1997).

The Oestroidea (Diptera: Calyptratae) is a group commonly accepted to be monophyletic (Griffiths, 1972; Hennig, 1973; McAlpine, 1989; Pape, 1992; Rognes, 1997). It is formally recognized to contain five other families besides Calliphoridae (McAlpine, 1989; Pape and Thompson, 2010): (1) Mystacinobiidae, with a single described species, *Mystacinobia zelandica*, which lives in close association with the bat *Mystacina tuberculata*; (2) Rhinophoridae (~170 spp.), whose members are, for the species of known biology, parasitoids of woodlice; (3) Oestridae (~170 spp.), whose all known species are parasites of mammals in their larval stage; (4) Tachinidae (~9600 spp.), one of the largest families in Diptera whose larvae are parasitoid of other arthropods; and (5) Sarcophagidae (~3000 spp.), a group whose larvae are mostly known for their necrophagous habit, although some of them are parasites of mammals or parasitoids of other invertebrates.

With the exception of Calliphoridae, all currently-recognized families of Oestroidea have well corroborated monophyletic status (Oestridae: Wood, 1987; Pape, 1992, 2001; Tachinidae: Rognes, 1986; Wood, 1987; Pape, 1992; Tschorsnig and Richter, 1998; Stireman et al., 2006; Sarcophagidae: Pape, 1992, 1996; Kutty et al., 2010), although the monophyly of Rhinophoridae has currently been corroborated only by larval characters (Pape, 1986, 1992; Pape and Arnaud, 2001).

The number of families in Oestroidea was recently increased by the promotion of Rhiniidae, formerly a subfamily of Calliphoridae with ~370 described species (Pape et al., 2011), which is now accorded valid family status in the Biosystematic Database of World Diptera (Pape and Thompson, 2010). Other calliphorid subfamilies had already been proposed to be elevated to family status, such as the Mesembrinellinae (Guimarães, 1977) and the Bengaliinae (Lehrer, 2005), although there is still much controversy with these classifications and further studies are required.

The works of Rognes (1997) and Kutty et al. (2010), the later combining the information of a broad range of molecular markers from a very large dataset of Calyptratae species, have provided evidence for non-monophyly of the Calliphoridae and proposed different placements for the para/polyphyletic groups. However, there is still a large number of different phylogenetic hypotheses for relationships between oestroid families and calliphorid subfamilies, some of them poorly supported, highlighting the need for further studies.

Additionally, as the work of Kutty et al. (2010) – the largest study available so far on this subject – could not comprise an extensive evaluation of the effects of different phylogenetic inference methods in the inferred trees, in particular the influence of different data partitioning strategies in a Bayesian framework, due to the prohibitive nature of the very large assembled dataset, a more diverse investigation on this subject is still lacking.

In this context, this work provides a molecular phylogenetic analysis of interfamilial relationships in the Oestroidea, with emphasis on the placement of some calliphorid subfamilies, based on both nuclear and mitochondrial molecular markers, using different approaches to phylogenetic inference. Taking advantage of the robustness of the Bayesian framework regarding the use of complex and parameter-rich models, different data partition strategies were evaluated using both sequence- and secondary structure-based substitution models, and their impacts on the estimated topologies and overall support of the trees were assessed. Implications for the accuracy of the phylogenetic inference process when using parameter-rich models were then discussed.

Results described here complement the work of Kutty et al. (2010), giving an independent evaluation of some of their results and, therefore, contributing to a deeper and more confident understanding of the phylogeny of Oestroidea.

2. Materials and methods

2.1. Specimens and DNA extraction

The 56 specimens used in the molecular phylogenetic analyses are listed in Table 1. Calliphorid subfamilies were classified according to Rognes (1986, 1991, 1997), including the Rhiniinae as a subfamily, which has now family status, recognizing its phylogenetic independence. The term Calliphoridae *sensu stricto* (Calliphoridae *s.s.*) was used here to designate the Calliphoridae excluding Rhiniinae and Mesembrinellinae, whereas Calliphoridae *sensu lato* (Calliphoridae *s.l.*) was used to designate the traditional clade composed by (Calliphoridae *s.s.* + Rhiniinae + Mesembrinellinae).

Taxon sampling included 8 of the 13 calliphorid subfamilies recognized by Rognes (1997) and 4 of the 6 oestroid families currently recognized (here excluding Rhiniidae) (McAlpine, 1989). Species of the superfamilies Muscoidea and Hippoboscoidea were used as outgroups.

DNA extraction for frozen specimens was carried out using a phenol/chlorophorm protocol (Infante and Azeredo-Espin, 1995), whereas for dried and ethanol-preserved specimens both the DNAzol reagent (Invitrogen) and the Spin Tissue Mini-Kit (Invitek) were used.

2.2. PCR amplification, DNA cloning and sequencing

Four DNA regions were amplified by PCR and sequenced: (1) the complete region of the internal transcribed spacer 2 (ITS2), (2) a portion of the 5′ region of the 28S ribosomal subunit, both from the nuclear ribosomal DNA (rDNA) cluster, (3) the 5′ region of the cytochrome c oxidase subunit I (COI) and (4) the 3′ portion of the 16S rDNA, both from the mitochondrial genome.

PCR reactions for the ITS2 region were conducted with 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 2 mM $MgCl_2$, 80 μM dNTPs, 0.4 μM 5.8S primer (5′-ATCACTCGGCTCGTGGGATTCGAT-3′), 0.4 μM 28S primer (5′-GTTAGTTTCTTTTCCTCCCCT-3′), 2.5U *Taq* DNA polymerase (Fermentas) and 1–2 μg of extracted DNA for a 25 μL reaction. PCR reactions were performed with an initial denaturation step of 95 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 45 s and 72 °C for 2 min, and a final elongation step of 72 °C for 3 min.

PCR amplifications for the remaining regions were conducted with 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1 mM $MgCl_2$, 80 μM dNTPs, 0.4 μM forward primer, 0.4 μM reverse primer, 2.5U *Taq* DNA polymerase (Fermentas) and 1–2 μg of extracted DNA for a

Table 1
Species used in the molecular phylogenetic analyses. Subfamilies of Calliphoridae were classified according to Rognes (1997).

Superfamily	Family	Subfamily	Species	Sequences (Genbank accession number)			
				ITS2	28S	COI	16S
Oestroidea	Calliphoridae	Chrysomyinae	*Chloroprocta idioidea* (Robineau-Desvoidy, 1830)	EF560180	JQ246603	JQ246658	JQ246708
			Chrysomya albiceps (Wiedemann, 1819)	EF560173	JQ246604	JQ246659	JQ246709
			Chrysomya bezziana (Villeneuve, 1914)	EF560174	JQ246605	JQ246660	JQ246710
			Chrysomya chloropyga (Wiedemann, 1818)	JQ246571	JQ246606	JQ246661	JQ246711
			Chrysomya megacephala (Fabricius, 1794)	EF560175	JQ246607	JQ246662	-
			Chrysomya putoria (Wiedemann, 1830)	EF560176	JQ246608	JQ246663	JQ246712
			Chrysomya rufifacies (Macquart, 1843)	EF560177	JQ246609	JQ246664	JQ246713
			Cochliomyia hominivorax (Coquerel, 1858)	EF560181	JQ246610	JQ246665	JQ246714
			Cochliomyia macellaria (Fabricius, 1775)	EF560182	JQ246611	JQ246666	JQ246715
			Hemilucilia segmentaria (Fabricius, 1805)	EF560192	JQ246612	JQ246667	JQ246716
			Hemilucilia semidiaphana (Rondani, 1850)	JQ246572	JQ246613	JQ246668	JQ246717
			Phormia regina (Meigen, 1826)	EF560190	JQ246614	JQ246669	JQ246718
			Protophormia terraenovae (Robineau-Desvoidy, 1830)	EF560193	JQ246615	JQ246670	JQ246719
		Calliphorinae	*Calliphora croceipalpis* (Jaennicke, 1867)	JQ246573	JQ246616	JQ246671	JQ246720
			Calliphora vicina (Robineau-Desvoidy, 1830)	EF560178	JQ246617	JQ246672	JQ246721
			Calliphora vomitoria (Linnaeus, 1758)	EF560179	JQ246618	JQ246673	JQ246722
		Toxotarsinae	*Sarconesia chlorogaster* (Wiedemann, 1830)	JQ246574	JQ246619	JQ246674	JQ246723
		Luciliinae	*Hemipyrellia* sp. (Townsend, 1918)[a]	JQ246575	JQ246620	JQ246675	JQ246724
			Hemipyrellia ligurriens (Wiedemann, 1830)	JQ246576	JQ246621	JQ246676	JQ246725
			Lucilia cuprina (Wiedemann, 1830)	EF560185	JQ246622	JQ246677	JQ246726
Oestroidea	Calliphoridae	Luciliinae	*Lucilia eximia* (Wiedemann, 1819)	EF560186	JQ246623	JQ246678	JQ246727
			Lucilia sericata (Meigen, 1826)	EF560187	JQ246624	JQ246679	JQ246728
			Lucilia sp. (Robineau-Desvoidy, 1830)[b]	JQ246577	JQ246625	JQ246680	JQ246729
		Auchmeromyiinae	*Auchmeromyia bequaerti* (Roubaud, 1913)	JQ246578	JQ246626	-	-
			Cordylobia anthropophaga (Blanchard & Berenger-Feraud, 1872)	JQ246579	JQ246627	JQ246681	JQ246730
			Hemigymnochaeta unicolor (Bigot, 1888)	JQ246580	JQ246628	JQ246682	JQ246731
			Pachychoeromyia praegrandis (Austen, 1910)	JQ246581	JQ246629	JQ246683	JQ246732
			Tricyclea sp. (Wulp, 1884)	JQ246582	JQ246630	JQ246684	JQ246733
		Bengaliinae	*Bengalia peuhi* (Villeneuve, 1914)	JQ246583	JQ246631	JQ246685	JQ246734
		Mesembrinellinae	*Eumesembrinella benoisti* (Séguy, 1925)	JQ246584	JQ246632	JQ246686	JQ246735
			Eumesembrinella quadrilineata (Fabricius, 1805)	JQ246585	JQ246633	JQ246687	JQ246736
			Mesembrinella bellardiana (Aldrich, 1922) ind.1	JQ246586	JQ246635	JQ246688	JQ246738
			Mesembrinella bellardiana (Aldrich, 1922) ind.2	EU076455	JQ246634	-	JQ246737
			Mesembrinella sp. (Giglio-Tos, 1893)[c]	EU076456	JQ246636	-	JQ246739
			Mesembrinella bicolor (Fabricius, 1805)	JQ246587	JQ246637	JQ246689	JQ246740
			Mesembrinella peregrina (Aldrich, 1922)	EF560188	JQ246638	JQ246690	JQ246741
		Rhiniinae[d]	*Cosmina fuscipennis* (Robineau-Desvoidy, 1830)	JQ246588	JQ246639	JQ246691	JQ246742
			Rhinia sp. (Robineau-Desvoidy, 1830)	JQ246589	JQ246640	JQ246692	JQ246743
			Rhyncomya soyauxi (Karsch, 1886)	JQ246590	JQ246641	JQ246693	JQ246744
			Thoracites sp. (Brauer & Bergenstamm, 1891)	JQ246591	JQ246642	JQ246694	JQ246745
	Sarcophagidae	Sarcophaginae	*Oxysarcodexia thornax* (Walker, 1849)	-	-	JQ246695	JQ246746
			Peckia ingens (Walker, 1849)	JQ246592	JQ246643	-	JQ246747
			Sarcophaga bullata (Parker, 1916)	JQ246593	JQ246644	JQ246696	JQ246748
Oestroidea	Tachinidae	Exoristinae	*Chetogena* sp. 1 (Rondani, 1856)	JQ246594	JQ246645	JQ246697	JQ246749
			Chetogena sp. 2 (Rondani, 1856)	JQ246595	JQ246646	JQ246698	JQ246750
			Tachinidae sp. (N/A)	JQ246597	JQ246648	-	JQ246752
		Dexiinae	*Prophorostoma pulchrum* (Townsend, 1927)	JQ246596	JQ246647	JQ246699	JQ246751
	Oestridae	Cuterebrinae	*Cuterebra* sp. (Clark, 1815)	JQ246598	JQ246649	JQ246700	JQ246753
			Dermatobia hominis (Linnaeus, 1781)	EF560183	JQ246650	JQ246701	JQ246754
Muscoidea	Muscidae	Muscinae	*Haematobia irritans* (Linnaeus, 1758)	EF560184	JQ246651	JQ246702	JQ246755
			Musca domestica (Linnaeus, 1758)	EF560189	JQ246652	JQ246703	JQ246756
			Stomoxys calcitrans (Linnaeus, 1758)	EF560191	JQ246653	JQ246704	JQ246757

		Cyrtoneurininae	*Cyrtoneuropsis maculipennis* (Macquart, 1843)	JQ246599	JQ246654	-	JQ246758
	Fannidae	Fanniinae	*Fannia* sp. (Robineau-Desvoidy, 1830)	JQ246600	JQ246655	JQ246705	JQ246759
Hippoboscoidea	Glossinidae	N/A	*Glossina morsitans* (Westwood, 1851)	JQ246601	JQ246656	JQ246706	JQ246760
	Hippoboscidae	Ornithomyinae	*Ornithoctona erythrocephala* (Leach, 1817)	JQ246602	JQ246657	JQ246707	JQ246761

[a] A female of *H. ligurriens* (Wiedemann, 1830) or *H. tagaliana* (Bigot, 1877).
[b] Ambiguous identification as *L. cuprina* (Wiedemann, 1830) or *L. sericata* (Meigen, 1826), due to incongruence between nuclear and mitochondrial genes (Tourle et al., 2009).
[c] Previously identified as *M. bellardiana* (Aldrich, 1922), needing further revision.
[d] The Calliphoridae subfamily Rhiniinae has been recently raised to its own family Rhiniidae (Pape and Thompson, 2010).

25 μL reaction. PCR reactions were performed with an initial denaturation step of 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 1 min and 72 °C for 2 min, and a final elongation step of 72 °C for 2 min. Forward and reverse primers for each gene region were: C1-N2320 (5′-AATCCTAATAATCCAATAGC-3′) and TW-J-1287 (5′-ACTAATAGCCTTCAAAGC-3′) for COI; 28S-F1 (5′-GGGAGGAAAAGAAACTAACAAGG-3′) and 28S-R1 (5′-CTGTTTCGGTCTTCCAT CAGGG-3′) for 28S; and LR-N-13398 (5′-CGCCTGTTTAACAAAAACAT-3) and L1R (5′-CCATTGCACTAATCTGCC-3′) for 16S. All PCR products were visualized by electrophoresis in 1× TAE (40 mM Tris–acetate, 1 mM EDTA) 1.0% agarose gels stained with ethidium bromide.

PCR products were purified using the Invisorb Fragment Clean-Up Kit (Invitek) and cloned into a pGEM-T Easy Vector (Promega). Competent DH5α *E. coli* cells were transformed using the $CaCl_2$ protocol (Sambrook et al., 1989) and then plated on LB medium with 50 mg/mL X-Gal and 50 μg/mL Ampicillin. Plates were grown at 37 °C overnight. Plasmids were extracted using an alkaline lysis protocol (Sambrook et al., 1989) and then digested with 5U *Eco*RI restriction enzyme (Invitrogen) at 37 °C for 2 h. Digestions were visualized by electrophoresis in 1× TAE 1% agarose gels, stained with ethidium bromide. At least three clones were submitted to sequencing reactions.

Automatic sequencing was conducted in an ABI 3700 (Applied Biosystems) using the "Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit" (Applied Biosystems), with the universal primers M13 Forward (5′-GTAAAACGACGGCCAG-3′) and M13 Reverse (5′-CAGGAAACAGCTATGAC-3′) and 200–600 ng of DNA.

2.3. Secondary structure prediction and sequence alignment

RNA structures of the *Drosophila melanogaster* 16S (Gutell et al., unpublished; de Rijk et al., 1997; Buckley et al., 2000; Cannone et al., 2002) and ITS2 (Young and Coleman, 2004) regions were used for homology modeling of both regions in Calyptratae. Since the 16S region is fairly conserved among the sampled taxa, and among insects as a whole (Buckley et al., 2000), the secondary structures of this region were established manually for each one of the species using *D. melanogaster*'s structure as a model. Secondary structures of the more variable helices H75 and H84 (Buckley et al., 2000) were predicted using the mfold v3.0 webserver (Zuker, 2003).

For the ITS2 region, secondary structure modeling was based on (1) homology-modeling based on the structure described for *D. melanogaster* (Young and Coleman, 2004) and for some calliphorid species (Marinho et al., 2011); (2) *in silico* prediction using the mfold v2.3 webserver (Zuker, 2003), with default parameters for folding and temperature set to 25 °C; and (3) comparison between structures predicted by mfold for all species to establish a common folding pattern to all sequences. This approach was employed since ITS2 is more variable with regard to both sequence and secondary structure, although a common pattern of folding can be established for all eukaryotes (Joseph et al., 1999). Sequence variability is, however, insufficient to obscure the phylogenetic signal in this region, as shown by Coleman (2003, 2007, 2009), Müller et al. (2007) and Marinho et al. (2011). A fuller discussion on the utility of the ITS2 region as a molecular marker for phylogenetic inference in Calliphoridae was provided by Marinho et al. (2011). Both ITS2 and 16S structures were annotated in the dot-bracket format and visualized using the VARNA software (Darty et al., 2009). Consensus secondary structures were determined using the SecondaryStructConsensus program of the PHASE 2.0 package (Gowri-Shankar and Jow, 2006).

Multiple sequence alignments of the 16S, COI and 28S regions were conducted using ClustalX 2.0 (Larkin et al., 2007), whereas for the ITS2 region sequence alignment accounting for secondary

structure information was carried out using the 4SALE software (Seibel et al., 2006). Except for the COI alignment, some manual adjustments were made on the final alignments.

2.4. Phylogenetic signal, substitution saturation and phylogenetic congruence analyses

Phylogenetic signal for each of the four gene regions analyzed was accessed using the parsimony-based method of Steel et al. (1993) and the entropy-based information method of Xia et al. (2003) and Xia and Lemey (2009), both implemented in DAMBE 5.2.38 (Xia, 2001; Xia and Xie, 2001). Steel's test was performed by sampling all possible quartets in the dataset and evaluated using the mean φ-value for each sequence (Xia and Lemey, 2009). Sequences with a mean φ-value smaller than 0.04 may be interpreted as lacking phylogenetic signal. Xia's test was performed with 10,000 replicates using only fully resolved sites.

Phylogenetic congruence was assessed using Concaterpillar 1.4 (Leigh et al., 2008), which uses hierarchical clustering and likelihood-ratio tests to detect congruence among datasets and evaluate if they can be combined by concatenation in the same dataset (null hypothesis of the topological congruence test) and if they can share estimated parameters (null hypothesis of the branch length congruence test) or if they must have parameters estimated and optimized separately. Concaterpillar 1.4 uses RAxML-VI-HPC (Stamatakis, 2006) for maximum-likelihood calculations.

2.5. Phylogenetic analyses

Model selection was carried out using MrAIC 1.4.4 (Nylander, 2004) for the complete concatenated dataset and for each one of its partitions that were used in the subsequent phylogenetic analyses.

Maximum parsimony (MP) analysis was conducted using TnT v1.1 (Goloboff et al., 2008) with the new technology search option (search at level = 50; initial addseqs = 15; find minimum tree length 10 times) and gaps treated as missing data. Node supports were measured by both bootstrap (BS) (1000 replicates) and jackknife (JK) resampling (1000 replicates; 36% independent character removal probability), using the same search options of the original search.

Non-partitioned maximum likelihood (ML) analyses were conducted using Garli v1.0 (Zwickl, 2006) (three independent replicates; 20,000,000 generations; 50 individuals per generation; default parameters for automated stopping) and PhyML 3.0 (Guindon and Gascuel, 2003) (all parameters estimated; tree topology search operations = SPR moves; add random starting trees = yes; number of random starting trees = 10) using for both the MrAIC 1.4.4 favored model for the four concatenated regions (GTR+I+G). Node supports were accessed using bootstrap (BS) (1000 replicates for both programs) and the SH-like test (SH) (PhyML 3.0 only).

Partitioned ML analysis was subsequently conducted using Garli v2.0 (Zwickl, 2006), with the same set of parameters described for the non-partitioned analyses. Each one of the four genetic regions were treated as different partitions and considered to evolve under its best fitted model (HKY+I+G for the ITS2 regions; GTR+I+G for the COI, 28S and 16S regions). Node supports were accessed using bootstrap (BS) resampling (100 replicates).

Bayesian inference (BI) analyses were carried out using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) and the mcmcphase software of the PHASE 2.0 package (Gowri-Shankar and Jow, 2006). The PHASE package was used in complementation to the MrBayes software as it implements more and different options of secondary structure-based models, which were then explored in the molecular phylogenetic analyses.

By default, each gene region was considered as a different partition, evolving under the best model found by MrAIC. ITS2 and 16S regions were further partitioned according to secondary structure conformation (single and double-stranded regions) and the COI region was partitioned according to codon position (1st, 2nd and 3rd position). As PHASE implements a large variety of secondary structure models (RNA models), the RNA7A model (the most general model in its category) was chosen for performing initial analyses since 7-state RNA models (which have a single state for all possible mismatches) are a reasonable intermediate between the more restricted 6-state models (which do not formally account for mismatches) and the more general 16-state models (which have separate states for all possible mismatches). After analyzing the results, the combination of models and partitions that resulted in the highest marginal log-likelihood value was further extended by testing the most general model in both the 6-state (RNA6A) and 16-state (RNA16A) model classes. Additionally, the RNA16I model was also evaluated since it considers base pair changes to occur in a two-steps process, like the Doublet model in MrBayes, instead of in a single step, as others RNA models implemented in PHASE. Table 2 summarizes all combinations of models and partitions tried.

In all analyses, each partition was allowed to have its own set of parameters with separate overall rates (unlink statefreq = (all) revmat = (all) shape = (all) pinvar = (all) and prset applyto = (all) ratepr = variable commands in MrBayes). For each combination of models and partitions, two independent analyses were run for 20,000,000 generations (sample frequency = 1000) and the burn-in was set to 25% after checking for convergence. Node supports were analyzed by their posterior probabilities (PP) in the resulting extended 50% majority rule consensus trees, in which clades having less than 50% PP are added to the final tree if they do not contradict previously established clades (default option in PHASE; sumt contype = allcompat option in MrBayes).

2.6. Bayesian-based model comparison

The different combinations of models and partitions used in the BI analyses were compared against each other and further evaluated using Bayes factors. For the comparisons, the harmonic mean of the likelihood values sampled during the stationary phase of the MCMC run was used as the model marginal-likelihood estimator, necessary for Bayes factor calculation, as suggested by Nylander et al. (2004). Interpretations for the Bayes factor results were based on the table presented by Kass and Raftery (1995).

To assess if any likelihood improvement in the Bayesian analyses was accompanied by significant changes in the estimated topologies, overall support of trees and inferred branch lengths, all Bayesian trees were (1) compared using symmetric distances (Robinson and Foulds, 1981), as implemented in the TreeDist software of the PHYLIP 3.67 package (Felsenstein, 2005); (2) evaluated by their average support, estimated as the arithmetic mean of the posterior probabilities of all nodes in each tree; and (3) compared by the total tree length (TL), the sum of all branch lengths, averaged using all trees sampled in the stationary phase of the MCMC run.

2.7. Likelihood-map analyses

After the phylogenetic analyses, conflicting phylogenetic hypothesis among methods were further evaluated using likelihood-maps (Strimmer and Von Haeseler, 1997), as implemented in the Tree-Puzzle 5.2 software (Schmidt et al., 2002). For these analyses, taxa were clustered in four groups based on the previously inferred phylogenetic trees and the percentage of quartets of species favoring each one of the three possible tree topologies were counted. Likelihood-map analyses were

Table 2
Summary of the different combinations of models and partitions used in the Bayesian inference analyses using MrBayes and PHASE. Numbers of free parameters are given, including branch lengths (last column).

Program	Model/partition combination	No. of partitions	Regions								No. of parameters (model/partitions)	No. of parameters (total)
			ITS2		COI			28S	16S			
			ss	ds	1st	2nd	3rd		ss	ds		
MrBayes	No structure/codon (NS/C)	6	HKY+I+G		GTR+I+G	HKY	HKY+G	GTR+I+G	GTR+I+G		51	160
	No structure/no codon (NS/NC)	4	HKY+I+G		GTR+I+G			GTR+I+G	GTR+I+G		40	149
	Structure/codon (S/C)	8	HKY+I+G	Doublet (GTR)+I+G	GTR+I+G	HKY	HKY+G	GTR+I+G	GTR+I+G	Doublet (GTR)+I+G	97[a]	206
	Structure/no codon (S/NC)	6	HKY+I+G	Doublet (GTR)+I+G	GTR+I+G			GTR+I+G	GTR+I+G	Doublet (GTR)+I+G	86[a]	195
PHASE	No structure/codon (N/SC)	6	HKY+I+G		TN93+I+G	HKY	HKY+G	GTR+I+G	GTR+I+G		48	157
	No structure/no codon (NS/NC)	4	HKY+I+G		GTR+I+G			GTR+I+G	GTR+I+G		40	149
	Structure/codon (S7A/C)	8	HKY+I+G	RNA7A+I+G	TN93+I+G	HKY	HKY+G	GTR+I+G	GTR+I+G	RNA7A+I+G	106	215
	Structure/no codon (S7A/NC)	6	HKY+I+G	RNA7A+I+G	GTR+I+G			GTR+I+G	GTR+I+G	RNA7A+I+G	98	207
	Structure/codon (S6A/C)	8	HKY+I+G	RNA6A+I+G	TN93+I+G	HKY	HKY+G	GTR+I+G	GTR+I+G	RNA6A+I+G	91[b]	200
	Structure/codon (S16A/C)	8	HKY+I+G	RNA16A+I+G	TN93+I+G	HKY	HKY+G	GTR+I+G	GTR+I+G	RNA16A+I+G	91[b]	200
	Structure/codon (S16I/C)	8	HKY+I+G	RNA16I+I+G	TN93+I+G	HKY	HKY+G	GTR+I+G	GTR+I+G	RNA16I+I+G	94[a]	203

[a] Both the Doublet model of MrBayes and the RNA16I model of PHASE consider base pair changes occurring through a two-step process and they have 16 frequency parameters and six rate parameters from the GTR model (20 free parameters).
[b] The RNA6A model has 15 rate parameters and six frequencies (with two constraints) resulting in 19 free parameters, the same number of free parameters of the RNA16A, which have 16 frequencies and five rate parameters (with two constraints).

performed evaluating all possible quartets of the dataset, with accurate parameter estimation (slow method) using the "Quartet sampling + NJ tree" option.

3. Results

3.1. Sequence and secondary structure analyses

The total PCR-amplified length and nucleotide content of each gene region used in the phylogenetic analyses are shown in Table S1 (Supplementary material). In both nuclear molecular markers (ITS2 and 28S) there was a considerable length variation in the amplified sequences, more pronounced in the oestrid species (*Cuterebra* sp. and *D. hominis*). All regions presented an A+T bias in their nucleotide content, which was greater in the ITS2 region.

Predicted secondary structures of ITS2 and 16S are shown in Figs. S1 and S2 (Supplementary material). With the exception of helices H75 and H84, following the nomenclature of Buckley et al. (2000), helix-loop regions of 16S were very conserved and could be easily modeled based on the *D. melanogaster* structure. While for helix H75 structures predicted by mfold were consistent along the sampled taxa (Fig. S1C), modeling of the most variable helix H84 was more difficult and the only two base pairs that are present in all structures are shown in Fig. S1A. Alternative foldings for this helix are shown in Fig. S1B.

Predicted secondary structures of ITS2 conform to the commonly accepted 4-domains model for Eukaryota (Joseph et al., 1999), as shown in Fig. S2. Structural organization can, however, be variable in each helix-loop region, remarkably in domain III, which is possibly branched in oestroid and muscoid species but not in hippoboscoid and acalyptrate species. Initial portions of helix-loop domains I, II and III are very conserved, as also are the sequence motif GUCUAGCAUA in the terminal portion of helix-loop domain III and the pyrimidine mismatch in domain II (Young and Coleman, 2004; Marinho et al., 2011), which is a C for most species.

The final concatenated, aligned dataset of the four gene regions, corrected by structural information for both 16S and ITS2 regions, is 3355 nucleotides. Individual alignments for the four regions are available as Supplementary material.

3.2. Phylogenetic signal, substitution saturation and phylogenetic congruence analyses

Phylogenetic signal analyses based on substitution saturation showed that, without further partitioning, all four molecular markers have experienced little substitution saturation and should possess enough information to infer phylogenetic relationships among the taxa of Calliphoridae *s.s.* (Table S2 – Supplementary material). For inferring phylogenetic relationships among taxa above this level, i.e., Oestroidea and Calyptratae, the 28S and 16S regions should be more suitable (mean φ for Steel's test >0.04), even though the ITS2 and COI regions did not present significant substitution saturation at this level when considering only fully resolved sites (Xia's test).

Partitioning the ITS2 and 16S datasets based on structural conformation (Table S2) led to a significant increase in the phylogenetic signal when considering only double-stranded regions, whereas no significant changes in the phylogenetic signal (based on the mean φ of Steel's test) were observed for single-stranded regions. Nevertheless, these regions have experienced a higher level of substitution saturation when compared to double-stranded regions. Partitioning the COI region by codon position led to a significant increase in phylogenetic signal when considering the first

and second positions, but not the third, which seems to have experience significant substitution saturation (Iss > Iss.c A in Xia's test). These results show that the phylogenetic signal presented in each gene region is to some extent limited by its less informative partition.

Topological congruence tests showed that all four gene regions can be concatenated in the same dataset for phylogenetic analyses ($p = 0.1198$ for concatenating ITS2 and 28S; $p = 0.0644$ for concatenating ITS2–28S and 16S; and $p = 0.1824$ for concatenating ITS2–28S–16S and COI), whereas the branch length congruence test showed that each gene region should have parameters estimated separately ($p < 0.05$ in all comparisons). These results, together with the results from the phylogenetic signal and substitution saturation analyses, suggest that data partitioning with separate parameter estimation could be an appropriate way for more fully explore the molecular dataset.

3.3. Phylogenetic analyses

Inferred phylogenetic trees revealed consistent relationships for most of the calyptrate groups analyzed (Figs. 1, S3, S4 and S5 – Supplementary material). A monophyletic Oestroidea clade, placed within a paraphyletic Muscoidea, was highly supported in the BI analyses (PP = 0.99/1.00; Figs. 1A and 2), moderately supported in the ML analyses (BS > 75, SH = 0.97; Figs. 1A, S3 and S4), and considerably less supported in the MP analysis (BS = 48/JK = 67; Figs. 1C and S5). With the exception of Calliphoridae *s.l.*, and Calliphoridae *s.s.* in the MP analysis, all families of Oestroidea were recovered as monophyletic with high support (MP – BS > 95; JK > 99/ML – BS > 95; SH > 0.97/BI – PP > 0.99). Monophyly of Calliphoridae *s.s.* was reasonably supported in the BI (average PP > 0.92) and ML (SH = 0.86) analyses, although in the later the BS support was significantly lower (non-partitioned analyses: Garli = 18/PhyML = 61; partitioned analysis: Garli = 46).

Relationships among families of Oestroidea were more variable, especially when considering the placement of Oestridae, which was recovered as (1) sister taxon of the remaining Oestroidea in the MP trees; (2) sister taxon of Rhiniinae + Sarcophagidae + Calliphoridae *s.s.* in the non-partitioned ML analyses and in some BI trees; and (3) sister taxon of Rhiniinae in the partitioned ML analysis and in most of the BI trees (Fig. 2). Sarcophagidae was recovered as sister taxon of Calliphoridae *s.s.* in the MP, ML and in all but one of the BI analyses (S/C analysis – run B), in which it was recovered as sister taxon of the Oestridae + Rhiniinae + Calliphoridae *s.s.* clade. For the Calliphoridae *s.l.* subfamilies not included in the Calliphoridae *s.s.* clade, Mesembrinellinae was consistently recovered as sister taxon of Tachinidae (MP – BS = 84, JK = 96; ML – BS > 79, SH = 0.95; BI – PP > 0.99), while Rhiniinae was recovered as sister taxon of Sarcophagidae + Calliphoridae *s.s.* in the MP, ML (non-partitioned analyses) and in some BI trees, although poorly supported (MP – BS = 67, JK = 86; ML – BS < 58, SH = 0.00; BI – average PP PHASE = 0.84), and as sister taxon of Oestridae in the ML (partitioned analysis) and in most of the BI analyses (ML – BS = 52; BI – average PP = 0.85).

All subfamilies of Calliphoridae *s.l.* were recovered as monophyletic (MP – BS = > 80, JK > 88; ML – BS > 74, SH > 0.96; BI – PP > 0.99). For the relationships among subfamilies of Calliphoridae *s.s.*, both ML and BI analyses supported a closer relationship between Bengaliinae and Auchmeromyinae (ML – Garli BS > 76, PhyML BS = 80, SH = 0.96; BI – average PP PHASE = 0.99, MrBayes = 0.97) and between Luciliinae, Toxotarsinae and Calliphorinae (ML – Garli BS > 86, PhyML BS = 90, SH = 0.97; BI – average PP PHASE = 0.99, MrBayes = 1.00). The Luciliinae + Toxotarsinae + Calliphorinae clade was recovered as sister taxa of Chrysomyinae in the non-partitioned ML analyses (Garli BS = 20, PhyML BS = 44, SH = 0.59) and that of Bengaliinae + Auchmeromyinae in the partitioned ML and BI analyses (ML – BS = 44; BI – average PP PHASE = 0.69, MrBayes = 0.66).

For relationships among genera of Chrysomyinae, the best sampled subfamily of Calliphoridae *s.s.* in the phylogenetic analyses, both ML and BI trees recovered a closer relationship between *Chrysomya* and *Phormia* + *Protophormia* (ML – Garli BS > 40, PhyML BS = 49, SH = 0.50; BI – average PP PHASE = 0.80, MrBayes = 0.89) and between *Cochliomyia* and *Hemilucilia* (ML – Garli BS > 55, PhyML BS = 62, SH = 0.62; BI – average PP PHASE = 0.95, MrBayes = 0.97). *Chloroprocta idioidea* was recovered as sister taxon of the remaining Chrysomyinae in the non-partitioned ML analyses and in the majority of the BI trees, while in some BI trees (MrBayes NS/NC and S/NC analyses) it was recovered as sister taxon of the *Cochliomyia* + *Hemilucilia* clade (average PP = 0.41) and in the partitioned ML analyses it was placed with the *Chrysomya* + (*Phormia* + *Protophormia*) clade (BS = 23). For relationships among genera of Auchmeromyinae, the other subfamily of Calliphoridae *s.s.* with more than two sampled genera, all inferred phylogenies support a closer relationship between *Hemigymnochaeta* and *Tricyclea* (MP – BS = 99, JK = 99; ML – BS = 100, SH = 1.00; BI – PP = 1.00) and between *Cordylobia*, *Auchmeromyia* and *Pachychoeromyia* (MP – BS = 99, JK = 99; ML – BS > 99, SH = 0.99; BI – PP > 0.99).

3.4. Bayesian-based model comparison

The Bayesian-based model comparison showed a significant improvement in marginal log-likelihood values as model complexity increased, with analyses considering both codon position and structural partitions presenting the highest log-likelihood values (Table 3A and B). In the expanded PHASE analyses, differing only in the complexity of the RNA model used, an opposite pattern was observed, as log-likelihood values decreased with increasing model complexity (Table 3C).

These significant changes in the log-likelihood values were accompanied by few topological changes, as revealed by the symmetrical distances shown in Table S3 (Supplementary material), some of them not necessarily correlated with the pattern of changing log-likelihood values. While different placements for the Sarcophagidae and for *Chloroprocta* (Chrysomyinae) were found in the MrBayes analyses (Table S4A – Supplementary material), in the initial PHASE analyses the only observed topological change was regarding the relative position of the rhiniine and oestrid clades (Table S4B – Supplementary material). The expanded PHASE analyses (Table S4C – Supplementary material) revealed some topological changes additional to those found in the initial analyses, including different relationships among species of Tachinidae and the sister group status of Oestroidea to *Fannia* sp. or Muscidae.

Average PP support of trees also showed little variation regarding the use of different partitioning strategies. In MrBayes analyses, average support showed a slight decrease as marginal log-likelihood increases (Table S3A), probably reflecting the increased topological uncertainty indicated by the number of trees included in the 99% credible set (NS/NC: [A] = 4008, [B] = 3952; NS/C: [A] = 4338, [B] = 4412; S/NC: [A] = 4378, [B] = 4479; S/C [A] = 4773, [B] = 4735). In PHASE analyses, changes in average support values were not directly correlated with changes in log-likelihood values, as analyses with intermediate log-likelihoods showed the highest average PP supports (S7A/NC in the initial PHASE analyses – Table S3B – and S7A/C in the expanded analyses – Table S3C).

Despite the small changes observed in the average support, some clades presented significant changes in support as model complexity altered (Table S4), especially in MrBayes analyses. While for some clades PP support values decreased as log-likelihood values increased, such as the support for monophyly of

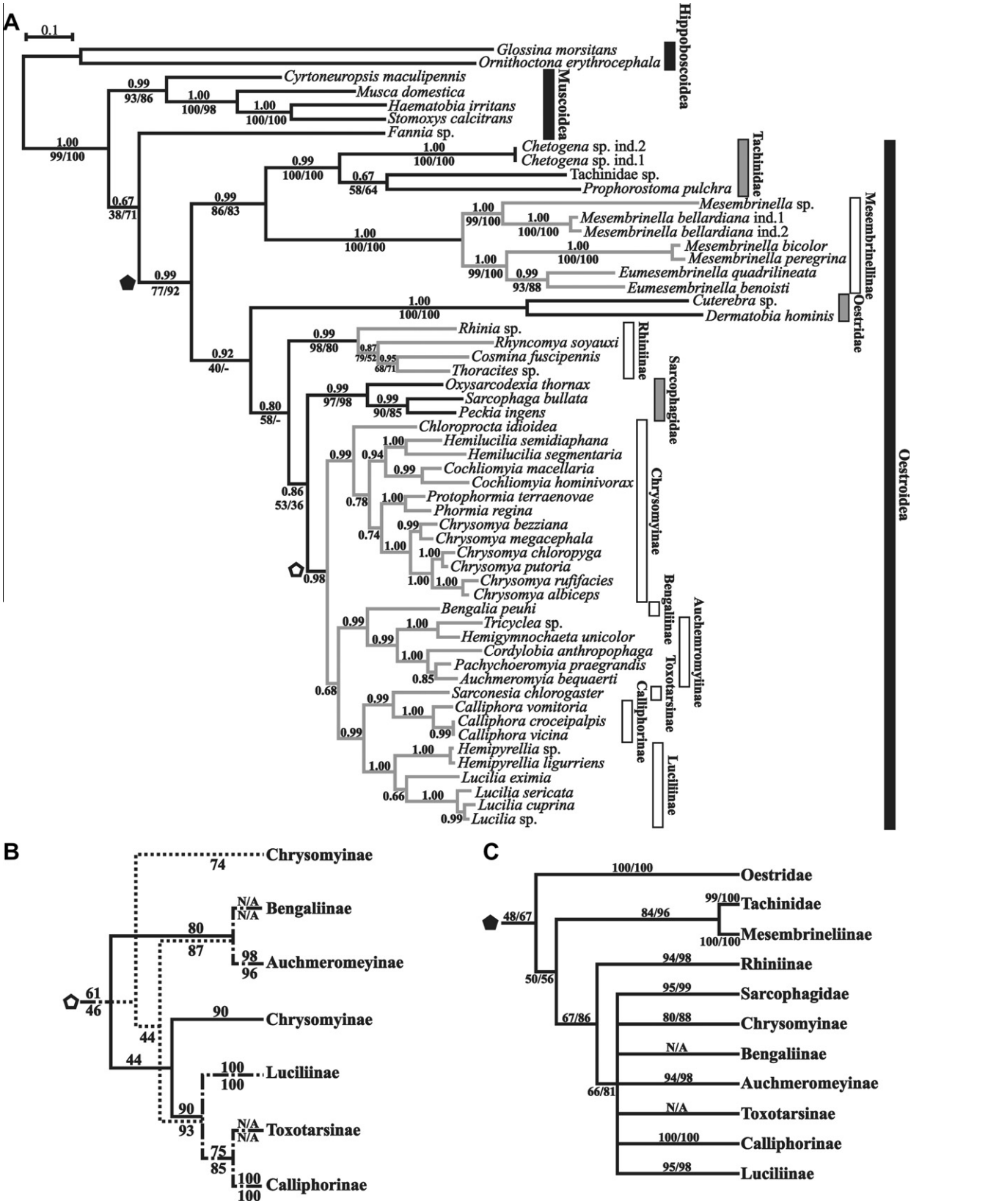

Fig. 1. (A) Bayesian tree inferred using PHASE with the S7A/C model/partition combination (see Table 2 for details). The tree topology is identical to the ML trees, with the exception of the relationships inside the Calliphoridae *s.s.* clade (both partitioned and non-partitioned ML analyses) and the placement of Rhiniinae (partitioned analysis only). Bayesian PP and maximum-likelihood BS supports (left = PhyML/non-partitioned; right = Garli/partitioned) are shown above and below branches, respectively. Clades formally attributed to the Calliphoridae are shown in gray. (B) Alternative resolution for the sub-familial relationships in the Calliphoridae *s.s.* clade, as inferred in the non-partitioned (continuous lines) and partitioned (dashed lines) ML analyses. Bootstrap support values for the non-partitioned (PhyML) and partitioned (Garli) analyses are shown above and below branches, respectively. The complete ML trees are shown in Figs. S3 and S4. (C) Interfamilial relationships in Oestroidea as inferred in the strict consensus tree of the 17 maximum-parsimony trees obtained with TnT. Bootstrap (left) and Jackknife (right) support values are given above the respective branches. The complete maximum-parsimony tree is shown in Fig. S4.

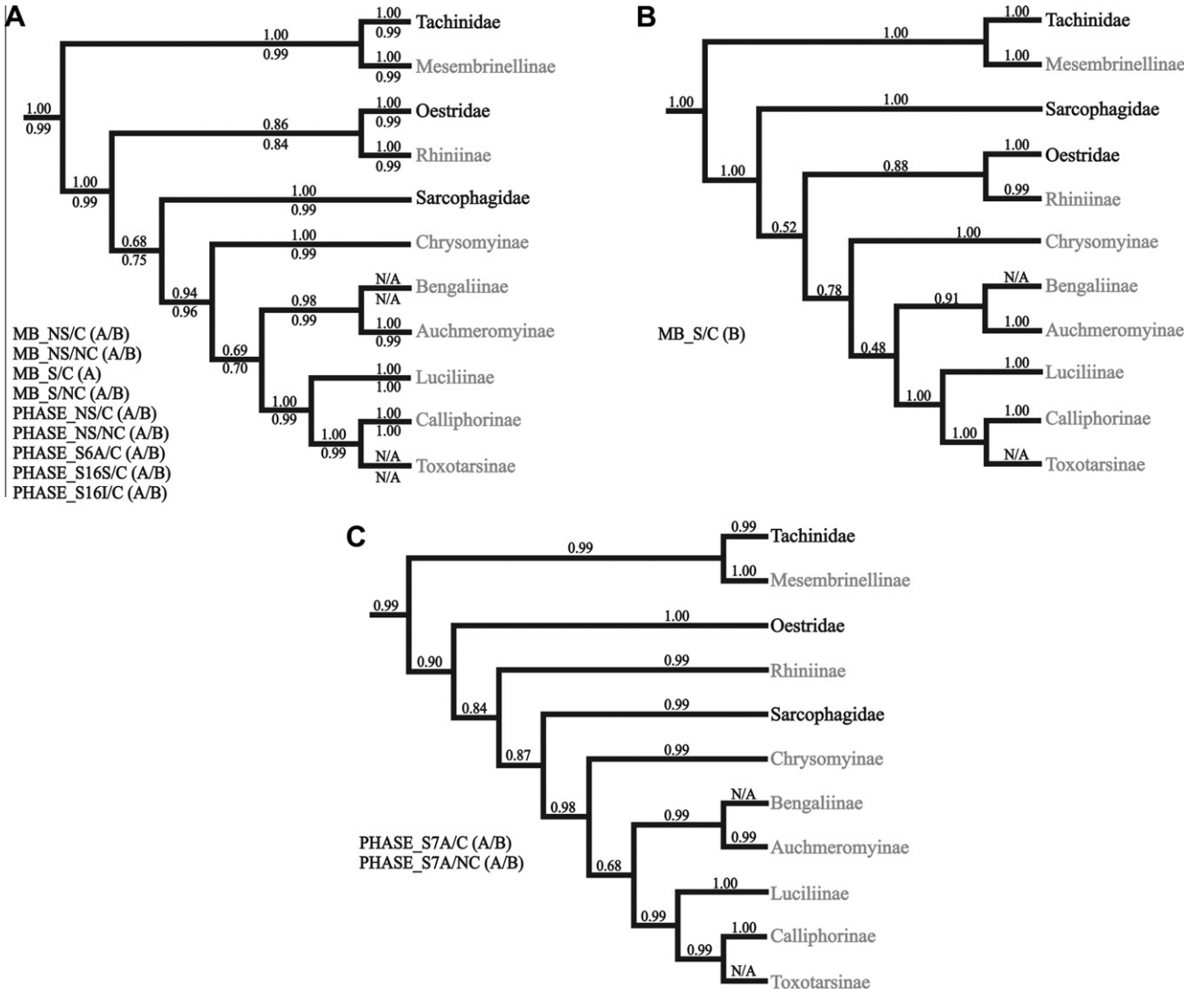

Fig. 2. Different hypothesis for interfamilial relationships in Oestroidea inferred by Bayesian analyses. (A) Topology recovered by the majority of the partitioning strategies used (detailed to the left). Average posterior probabilities for MrBayes and PHASE conducted analyses are shown above and below all branches, respectively. (B) Relationships inferred in one of the two independent Bayesian inference runs using the model/partition combination S/C (see Table 2 for details). (C) Topology recovered in the Bayesian analyses using the S7A/C and S7A/NC model/partition combinations in PHASE. Names in gray are clades traditionally placed in Calliphoridae.

Calliphoridae *s.s.* in MrBayes analyses (Table S4A) and for the sister taxa status of Oestroidea + *Fannia* sp. in both MrBayes and PHASE analyses (Tables S4A and S4B), other clades seemed to be more sensitive to the inclusion or omission of separate partitions for different codon positions. In this sense, PP support values for some clades were higher when codon positions are not allowed to evolve under different models [e.g. (*Chrysomya* + (*Phormia*, *Protophormia*)) and (*L. eximia* + (*L. sericata*, *L. cuprina*, *Lucilia* sp.))] while for others they are higher when different codon position partitions are considered [e.g. ((*Chrysomya*, (*Phormia*, *Protophormia*)) + (*Cochliomyia*, *Hemilucilia*))].

Allowing different codon positions to evolve at distinct rates also had some influence in the overall estimated tree length, at least in the MrBayes analyses (Table S3A). In these analyses, those accounting for different codon position partitions resulted in shorter trees and the only directly correlated topological changes were the placement of *C. idioidea* as sister group of (1) the remaining Chrysomyinae (in the NS/C and S/C analyses and as recovered in all other analytical methods) or (2) the *Cochliomyia* + *Hemilucilia* clade, with the former relationship achieving higher PP support. In the PHASE analyses, although more discrete changes in tree length were observed, branch lengths seemed to be more influenced by model complexity, as they showed a slight increase as model complexity increases (Tables S3B and S3C).

3.5. Likelihood-map analyses

Likelihood-map analyses (Fig. 3) showed that although the final concatenated dataset seemed to have enough resolution for solving relationships in the three hierarchical levels analyzed (5% or less of fully unresolved quartets) (Fig. 3A, B and E), there is still some conflicting signal considering both the relative position of Oestridae, Rhiniinae, Calliphoridae *s.s.* + Sarcophagidae and Tachinidae + Mesembrinellinae (Fig. 3C) and of Luciliinae + Toxotarsinae + Calliphorinae, Chrysomyinae, Bengaliinae + Auchmeromyinae and Sarcophagidae (Fig. 3F). In the latter case, this conflicting signal can be partially explained by incongruence among the information contained in each one of the four molecular markers used (Fig. 3G), although for both cases the conflict can be better explained by lack of resolution to solve these two particular relationships based on the analyses of the sampled molecular markers.

Table 3
Results of the Bayes factor comparison analyses. (A) MrBayes analyses. (B) Initial PHASE analyses. (C) Expanded PHASE analyses using the different RNA models implemented in the software. Significant differences are shown in emboldened numbers. Positive values indicate support for M_1 (row models) over M_0 (column models), while negative values indicate support for M_0 over M_1.

Marginal Log*L* (harmonic mean)	Method		M_0							
			NS/C (run A)	NS/C (run B)	NS/NC (run A)	NS/NC (run B)	S/C (run A)	S/C (run B)	S/NC (run A)	S/NC (run B)
A										
−26975.96863	M_1	NS/C (run A)	0							
−26975.97567		NS/C (run B)	−0.0140	0						
−27232.69321		NS/NC (run A)	**−513.4491**	**−513.4350**	0					
−27232.40883		NS/NC (run B)	**−512.8804**	**−512.8663**	0.5687	0				
−26611.45897		S/C (run A)	**729.0193**	**729.0334**	**1242.4684**	**1241.8997**	0			
−26611.42615		S/C (run B)	**729.0849**	**729.0990**	**1242.5341**	**1241.9653**	0.0656	0		
−26868.27385		S/NC (run A)	**215.3895**	**215.4036**	**728.8387**	**728.2699**	**−513.6297**	−513.6954	0	
−26868.47684		S/NC (run B)	**214.9835**	**214.9976**	**728.4327**	**727.8639**	**−514.0357**	−514.1013	−0.4059	0
			M_0							
			NS/C (run A)	NS/C (run B)	NS/NC (run A)	NS/NC (run B)	S7A/C (run A)	S7A/C (run B)	S7A/NC (run A)	S7A/NC (run B)
B										
−26662.21019	M_1	NS/C (run A)	0							
−26661.64489		NS/C (run B)	1.1306	0						
−27197.83687		NS/NC (run A)	**−1071.2533**	**−1072.3839**	0					
−27197.78172		NS/NC (run B)	**−1071.1430**	**−1072.2736**	0.1103	0				
−26044.03051		S7A/C (run A)	**1236.3593**	**1235.2287**	**2307.6127**	**2307.5024**	0			
−26043.16646		S7A/C (run B)	**1238.0874**	**1236.9568**	**2309.3408**	**2309.3405**	1.7281	0		
−26575.05535		S7A/NC (run A)	**174.3096**	**173.1790**	**1245.5630**	**1245.4527**	**−1062.0496**	**−1063.7777**	0	
−26573.22752		S7A/NC (run B)	**177.9653**	**176.8347**	**1249.2187**	**1249.1084**	**−1058.3940**	**−1060.1221**	3.6556	0
			M_0							
			S6A/C (run A)	S6A/C (run B)	S7A/C (run A)	S7A/C (run B)	S16A/C (run A)	S16A/C (run B)	S16I/C (run A)	S16I/C (run B)
C										
−25470.42685	M_1	S6A/C (run A)	0							
−25474.80717		S6A/C (run B)	−8.7606	0						
−26044.03051		S7A/C (run A)	**−1147.2073**	**−1138.4466**	0					
−26043.16646		S7A/C (run B)	**−1145.4792**	**−1136.7185**	1.7281	0				
−26320.51977		S16A/C (run A)	**−1700.1858**	**−1691.4252**	**−552.9785**	**−554.7066**	0			
−26324.70656		S16A/C (run B)	**−1708.5594**	**−1699.7987**	**−561.3521**	**−563.0802**	−8.3735	0		
−26333.09095		S16I/C (run A)	**−1725.3282**	**−1716.5675**	**−578.1208**	**−579.8489**	**−25.1423**	**−16.7687**	0	
−26333.65605		S16I/C (run B)	**−1726.4584**	**−1717.6977**	**−579.2510**	**−580.9791**	**−26.2725**	**−17.8989**	−1.1302	0

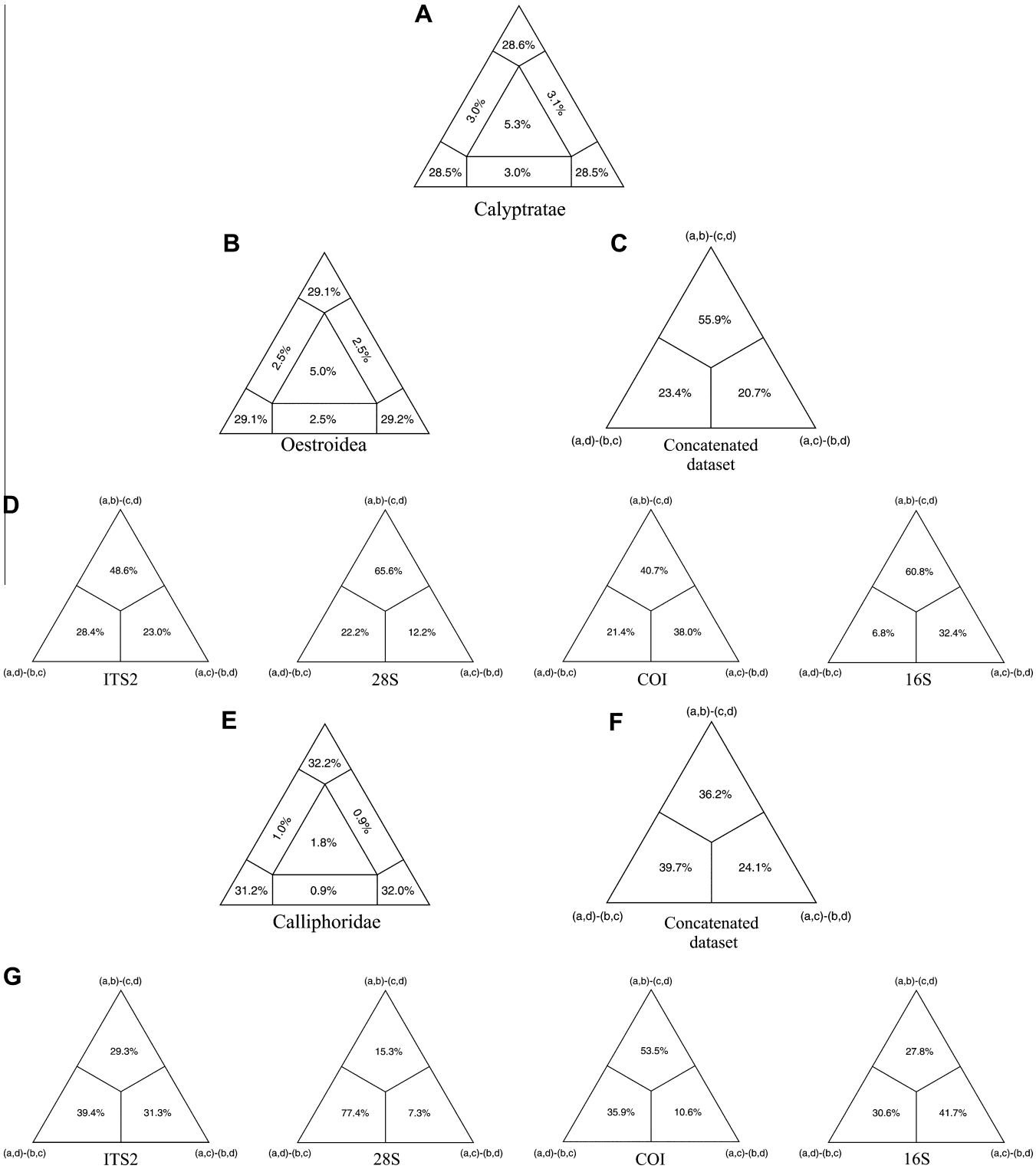

Fig. 3. Results of the likelihood-map analyses. (A, B and E) Resolution power of the complete concatenated dataset for analyses of the Calyptratae, Oestroidea and Calliphoridae *s.s.* groups, as recovered by analyzing all possible quartets of species in the dataset. The center triangle indicates the percentage of fully unresolved quartets while the vertices indicate the percentages of fully resolved quartets. (C) Percentage of quartets favoring one of the three possible previously defined relationships among clades: a = Calliphoridae *s.s.* + Sarcophagidae; b = Rhiniinae; c = Oestridae; d = Tachinidae + Mesembrinellinae. The complete concatenated dataset was used. (D) The same analysis of (C) but considering individual gene regions. (F) Percentage of quartets favoring one of the three possible previously defined relationships among clades: a = Chrysomyinae; b = Calliphorinae + Luciliinae + Toxotarsinae; c = Auchmeromyinae + Bengaliinae; d = Sarcophagidae. (G) The same analysis of (F) but considering individual gene regions.

4. Discussion

4.1. Phylogenetic analyses

The monophyletic status of Oestroidea was recovered, as previously shown by Griffiths (1972), Hennig (1976), McAlpine (1989), Pape (1992) and Rognes (1997). Its placement inside a non-monophyletic muscoid grade, as recently suggested by Kutty et al. (2008), was also confirmed. With the exception of Calliphoridae *s.l.*, all other families of Oestroidea were recovered as monophyletic, but the small number of sampled species in these families allows no further interpretation.

Regarding interfamilial relationships in the Oestroidea, most of the inferred topologies obtained here are new phylogenetic hypotheses for the group. As there is always the question of differences in taxon sampling and in the number and nature of sampled markers (especially in the case of morphological versus molecular characters), some of the results presented here are difficult to discuss and interpret relative to currently available phylogenies for the group.

In the results presented here, both ML and BI trees suggested a split of Oestroidea into two clades, one of them composed of Tachinidae + Mesembrinellinae and the other one of Oestridae + Rhiniinae + Sarcophagidae + Calliphoridae *s.s.* MP analysis, although recovering a similar overall relationship, suggested a placement of Oestridae as sister group of the remaining Oestroidea and a polyphyly among Sarcophagidae and Calliphoridae *s.s.* subfamilies, suggesting a very close relationship between these two families.

The close relationship between Tachinidae and Mesembrinellinae, as shown here and previously suggested in the molecular analyses of Kutty et al. (2010), does not corroborate previously published morphology-based phylogenetic relationships for the group, in which Tachinidae was recovered as (1) sister group of Rhinophoridae (family not represented in this study) and closely related to Oestridae (McAlpine, 1989); (2) sister group of Sarcophagidae and probably closely related to Rhiniinae (Rognes, 1997); or (3) sister taxa of Sarcophagidae or Oestridae (Pape, 1992: the clade Tachinidae + Oestridae was recovered only when character 32 – Dorsolateral phallic processes, apical configuration – was removed from the analyses).

The placement of Mesembrinellinae apart from Calliphoridae is not broadly supported, especially by the morphological studies available so far, although some authors have already noticed some aberrant features in this group when comparing it with the remaining Calliphoridae (Hall, 1948). Guimarães (1977) argued for placing this group in its own family (Mesembrinellidae) based mostly in the non-telescopic ovipositor, the developed subscutellum (cf. Tachinidae) and the occurrence of macro- (or uni-) larviparous reproduction (Toma and Carvalho, 1995), the last a habit shared with the subfamily Ameniinae, which probably form a monophyletic clade with Mesembrinellinae (Rognes, 1997). On the other hand, Rognes (1986) stated that Mesembrinellinae exhibits all of the apomorphies of Calliphoridae used in his studies and that its subfamily status should thus be maintained.

As more molecular data become available, the newly inferred phylogenetic hypotheses for the group corroborate the taxonomic propositions of Guimarães (1977), although with a different topology from that which he initially proposed (sister taxon of Calliphoridae). Additional studies, including richer taxonomic sampling in the Mesembrinellinae and a combined approach with morphological and molecular data, are nevertheless necessary before further conclusions be drawn about this subject, although the need for a systematic revision in this group seems more than desirable.

The correct placement of Oestridae in the phylogeny of the Oestroidea seems to be a problematic issue, as this family appears to have undergone profound differentiation of both molecules and morphology, which has led to a highly specialized parasitic habit and rendered many comparisons of characters difficult. In the tree topologies inferred here, this family was the one with the highest number of different placements when different analytical methods were used. Its sister taxon status to the remaining Oestroidea, as recovered in the MP analyses, was also found by Kutty et al. (2010) in their ML and BI analyses. The alternative placements (sister group of Rhiniinae and sister group of Rhiniinae + Sarcophagidae + Calliphoridae *s.s.*) were consistently different to those proposed previously (McAlpine, 1989: sister group of Tachinidae + Rhinophoridae; Pape, 1992: sister group of Calliphoridae; Rognes, 1997 and Pape and Arnaud, 2001: inside Calliphoridae; Kutty et al., 2010: within a clade composed by Mesembrinellinae + Tachinidae).

The recently proposed exclusion of Rhiniinae from Calliphoridae, which was then placed in its own family, Rhiniidae, was corroborated here, although both inferred placements (as sister taxon to Oestridae or to Sarcophagidae + Calliphoridae *s.s.*) were significantly different from those currently published (Pape, 1992: inside Calliphoridae, next to Toxotarsinae and Chrysomyinae; Rognes, 1997: sister taxon of Sarcophagidae + Tachinidae; Pape and Arnaud, 2001: sister taxon of Rhinophoridae; Kutty et al., 2010: sister taxon of all Oestroidea except Sarcophagidae and Mystacinobiidae).

The closer relationship between Sarcophagidae and Calliphoridae *s.s.*, although not widely corroborated, has been previously proposed by McAlpine (1989), with both families in a clade that also includes the monotypic family Mystacinobiidae (not sampled here). Most previous studies suggested a closer relationship between the Sarcophagidae and the Tachinidae (Pape, 1992; Rognes, 1997; Tachi and Shima, 2010) or its placement, along with Mystacinobiidae, as sister group to the remaining Oestroidea (Kutty et al., 2010). For the Calliphoridae, most commonly proposed relationships placed this family, albeit with different compositions, closer to the Oestridae and Rhinophoridae (Tschorsnig, 1985; Pape, 1992; Rognes, 1997) or as sister taxon of the remaining Oestroidea (Griffiths, 1972).

The monophyletic clade of Calliphoridae recovered here (Calliphoridae *s.s.*) has three well-defined internal clades (1) Chrysomyinae (Ch); (2) Bengaliinae + Auchmeromyiinae (B+A); and (3) Luciliinae + Toxotarsinae + Calliphorinae (L+T+Ca). The overall relationships among these three clades is unclear because the relationship (Ch, (B+A, L+T+Ca)) is favored by some traditional classifications (which included both Bengaliinae and Auchmeromyiinae inside a broadly conceived subfamily Calliphorinae – (Hennig, 1973; Shewell, 1987)), while other published morphological phylogenetic analyses suggested the relationship (B+A, (Ch, L+T+Ca)) (including or excluding Oestridae in this clade: Pape, 1992; Rognes, 1997; Pape and Arnaud, 2001) and the molecular analyses of Kutty et al. (2010) suggested (L+T+Ca, (Ch, B+A)) (in fact, Bengaliinae was included inside a paraphyletic Chrysomyinae). In our analyses, while the partitioned ML and BI trees favored the relationship (Ch, (B+A, L+T+Ca)), the non-partitioned ML trees suggested (B+A, (Ch, L+T+Ca)), neither highly supported. As shown in the likelihood-map analyses, even though the concatenated dataset gives a slightly higher support for the grouping of (B+A) and (L+T+Ca) (Fig. 3F), there is still too much conflict among the individual genes (Fig. 3G) to drawn any firm conclusion in this subject.

The monophyly of Auchmeromyiinae + Bengaliinae is well corroborated by previous analyses (Rognes, 1997), as is also the relationships among genera recovered within Auchmeromyiinae (*Cordylobia* + *Auchmeromyia* + *Pachychoeromyia* and *Tricyclea* + *Hemigymnochaeta* – Zumpt, 1956).

The close relationship between the subfamilies Calliphorinae and Luciliinae is also well corroborated (Kutty et al., 2010) and in some classifications they are indeed included as tribes in the same subfamily, Calliphorinae, defined in a broad sense (Hennig, 1973; Shewell, 1987; Kurahashi, 1989). The inclusion of Toxotarsinae in this clade seems to be corroborated only by molecular analyses (Kutty et al., 2010), as most morphological data suggest its placement closer to the Chrysomyinae (Boyes and Shewell, 1975; Pape, 1992 – including Rhiniinae; Rognes, 1997).

The monophyly of the Chrysomyinae was corroborated, as recently shown by Singh and Wells (2011), although Kutty et al. (2010) found it to be paraphyletic due to the inclusion of *B. peuhi* (Bengaliinae) in this clade. Relationships among genera are mostly in agreement with the ones described by Singh and Wells (2011), in which there seems to be no support for the current tribal classification in this group (Hall, 1948), as *Chrysomya* was found to be more closely related to genera of the Phormiini rather than to the remaining Chrysomyini (as first suggested by Rognes (1991)). The only conflicting relationship inferred here is the placement of *C. idioidea* as sister group of the remaining Chrysomyinae, because Singh and Wells (2011) found it to be placed among the Chrysomyini, excluding *Chrysomya*. *Chloroprocta* was recovered as sister taxon to *Hemilucilia* + *Cochliomyia* only in the MrBayes trees inferred without considering codon position partitions, with this relationship being very weakly supported (PP < 0.50 – Table S4A).

4.2. Bayesian-based model comparison

The phylogenetic information of each gene region seems to be limited by its less informative subpartition, since adding further partitions to these regions (either by structural conformation or codon position) resulted in a significant increase in the phylogenetic signal of at least one of the subpartitions, while the less informative one (i.e., the one with the highest substitution saturation index) maintains the same overall phylogenetic signal of the entire region. Thus, considering subpartitions in gene partitions and allowing them to evolve under their own best fitted models resulted in a best fit of the overall model/partition combination to the data, hence the significant increase in marginal log-likelihoods as model complexity increases (Table 3).

However, as model complexity increases, so does the overall uncertainty across the estimated parameters, including tree topology. In this sense, it is expected that the number of trees contained in the credible interval set increases as the number of model parameters increase (Nylander et al., 2004). With larger credible sets, more trees corroborating different phylogenetic hypothesis are included, ultimately resulting in poorly supported and sometimes unstable inferred topologies. This might explain the decrease in the overall PP support of trees as model complexity (and log-likelihood values) increases in both MrBayes and initial PHASE analyses (Tables S3A and S3B).

However, unexpectedly, the number of trees in the credible set for the MrBayes analyses seems to increase in proportion to the number of partitions considered in the dataset rather than in proportion to the number of parameters itself (as shown by Nylander et al. (2004)), as both models with intermediate complexity (S/NC and NS/C) have the same number of partitions and similar numbers of trees in the credible set even though the S/NC model is considerably more rich in parameters (86 free parameters) than the NS/C model (51 free parameters).

In the case of very parameter-rich models, tree topology uncertainty may be large enough to render inferred topologies very unstable and two independent runs performed in the same dataset under the same conditions (but with different random seeds) may result in distinct topologies with very similar log-likelihood values (as for the S/C MrBayes analyses).

Altering the structural and/or codon position partitions in the analyses seems to have small specific effects in terms of inferred topologies, total tree length and overall PP support of trees compared to the effects caused by increased model complexity and estimation uncertainty. Exceptions can be seen in the changes of PP support values for some particular clades and in the topological modifications in the placement of Oestridae (when including structural partitions) and *C. idioidea* (when including codon position partitions), the latter example including changes in estimated branch lengths. These small, specific observations contradict, to some extent, the common belief that considering partitioning strategies that more realistically depict and model the evolution of different gene regions should enhance phylogenetic estimation, leading to better estimated phylogenies (as suggested by the evaluated log-likelihood values).

However, as shown by Letsch and Kjer (2011) in their analyses of the effects of the use of different mixed DNA/RNA models in phylogenetic estimation of large Metazoan groups using rDNA sequences, when no significant substitution saturation is detected in the dataset (mainly in single-stranded regions), the use of a simple DNA model or a mixed DNA/RNA models should result in potentially correct, similar trees. It is then noteworthy that, even then, the use of mixed DNA/RNA models should result in better estimates of support for clades in the tree. It is possible that some of these conclusions can be extended to the consideration of codon position partitions too.

As also shown by Letsch and Kjer (2011), when significant substitution saturation is present in the dataset, simple DNA models and mixed DNA/RNA models with less parameter-rich RNA models (e.g., 6-state RNA models) should be preferred over mixed models with parameter-richer RNA models (e.g., 7- and 16-state RNA models). Although this is not precisely the case here, log-likelihood values indeed indicated a better performance of the RNA6A model over the RNA7A, RNA16A and RNA16I models (Table 3C). Nevertheless, special attention should be paid to the RNA7A model, as this model, among the ones used in the expanded PHASE analyses, was the only one to result in some topological changes (in the position of Oestridae) and the trees inferred under this model were the ones with the highest overall PP values (Table S4C). This may indicate that there is some phylogenetic information in the RNA molecules that is only taken into account when mismatched states are formally considered, which is not the case in the RNA6A model. However, as exploration of mismatched states in the RNA secondary structure molecule is expanded, attributing each mismatch state to its own category (as in the 16-state RNA models), overparameterization may obscure the phylogenetic information, hence the lower likelihood values of these models when compared to the 6- and 7-state models (Table 3C).

The complexity of the overall scenario depicted by the results obtained here makes impossible to conclude which model and partition combination may result in better estimated phylogenies in a Bayesian framework for this dataset. This is because the effects of different combinations in the BI analyses seems to depend on the balance between (1) applying more sophisticated partitioning strategies to more realistically model the evolution of structural RNA and protein-coding gene regions and (2) the ultimate results of overparameterization and increased uncertainty in the final estimated phylogeny. However, performing a deeper exploration in the analyses of multigene datasets actually gives important clues about (1) which relationships may be unstable and show differences in the placement of subordinated groups depending on the chosen method of analyses; (2) which clades are sensitive to increased model complexity and uncertainty in the phylogenetic estimation process and may be more or less supported under these conditions; and (3) which clades in the inferred tree need additional taxon and/or molecular marker samples to be placed more confidentially.

5. Conclusions

Interfamilial relationships in the Oestroidea are still far from being confidently defined and there remains a need for a better understanding of the affinities of the groups traditionally included in Calliphoridae. However, as more molecular phylogenetic studies for this group become available, more and new hypothesis are generated, some of them contradicting previously proposed phylogenies based on the analyses of morphological characters. Although this contradiction can be partially explained by differences in taxonomic sampling between these studies, some relationships may indeed be differentially inferred due to the distinct nature of the data, which emphasizes the importance of a unified phylogenetic approach, including both molecular and morphological characters, to elucidating relationships in this group.

Inferring phylogenetic relationships in this group can be particularly difficult as Schizophora (the group containing the Calyptratae and the Oestroidea) seems to have experienced a rapid, episodic radiation around 65 Mya (Wiegmann et al., 2011), which may have left little and sometimes conflicting signal depending on the molecular markers sampled. Moreover, the existence of some groups (families) of Oestroidea that might have experienced very different evolutionary rates, some of them related to a profound specialization and modification towards a better adaptation to parasitism (e.g. Oestridae), may render inferred phylogenies unstable and sometimes unreliable due to the problem of long branch attraction. Analyses using models that account for heterogeneous evolutionary rates among lineages may ameliorate this problem, but the large computational power required for these analyses may make employing them unfeasible for large datasets.

Based on the findings of this study, we provided additional evidence corroborating the non-monophyly of Calliphoridae. The recently exclusion of the former calliphorid subfamily Rhiniinae, which has been promoted to family rank (Rhiniidae), was confirmed, although its precise placement in the phylogeny is still uncertain. The placement of Mesmebrinellinae within Calliphoridae is dubious and further analyses are necessary to resolve this matter. Our results support Guimarães's (1977) proposal that this group should be placed apart from Calliphoridae, probably in its own family (Mesembrinellidae), and endorse Crosskey's (1965) view that an improved classification in Oestroidea "would probably result if the peculiar groups such as Mesembrinellinae were treated as families".

Evaluation of the effects of different partitioning strategies in the phylogenetic estimation process showed that the use of more complex, more parameter-rich models, although resulting in a better overall fit of the model to the data, does not necessarily leads to better estimated phylogenies. This result probably arises from the fact that, with increasing model complexity, there is also increasing uncertainty across the larger suite of estimated parameters, including the inferred tree topology. Moreover, when there is no significant level of substitution saturation in the dataset, little or no topological change could be observed in the inferred phylogenies under models with different complexities. In these cases most partitioning strategies should result in similar topologies, although with differentially supported clades. Thus, the use of models with increasing complexity should result, if not in better estimated topologies, at least in trees that are more properly supported, eliminating over- or under-estimated support for some clades.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.08.007.

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