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The first phylogenetic study of Mesembrinellidae (Diptera: Oestroidea) based on molecular data: clades and congruence with morphological characters

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Abstract

The Mesembrinellidae (Diptera: Oestroidea) comprise a small group of strictly Neotropical calyptrate flies, with 36 described species. The group has often been treated as a subfamily of Calliphoridae, but there is growing evidence that it corresponds to a distinct Oestroidea lineage. Internal relationships have so far been addressed based only on morphology, with results lacking resolution and support. This is the first molecular phylogeny for the group, which is based on the analyses of 80 terminal taxa (22 mesembrinellid and 28 outgroup species) and 5 molecular markers (*ITS2*, *28S*, *COI*, *COII* and *16S*). Maximum-parsimony, maximum-likelihood and Bayesian inference methods were used, the latter two with partitioning strategies considering codon position and secondary structure information. Results corroborate the Mesembrinellidae as a monophyletic lineage inside Oestroidea. Three clades were consistently recovered: (1) (*Laneella* + *Mesembrinella patriciae*); (2) (*Mesembrinella* (excluding *M. patriciae*) + *Eumesembrinella*); and (3) (*Huascaromusca* + *Giovanella*). Re-examination of the female reproductive tract of *M. patriciae* revealed a *Laneela*-type spermatheca, which corroborates the position of the species recovered in the molecular phylogenetic analyses. *Mesembrinella* and *Huascaromusca* are in all cases paraphyletic with regards to *Eumesembrinella* and *Giovanella*, respectively. These latter two genera should, thus, be seen as subjective junior synonyms.

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The group comprising *Mesembrinella* and related genera (Diptera: Calyptratae: Oestroidea) currently includes 36 described species distributed in nine genera (Guimarães, 1977; Bonatto, 2001; Bonatto and Marinoni, 2005; Wolff, 2013; Wolff et al., 2013, 2014). The entire clade is restricted to the Neotropical region (Table 1). The small number of species currently recognized in the group is probably the result of a historical neglect and recent collecting efforts and revisions of entomological collections have led to an increasing number of described species (Bonatto and Marinoni, 2005; Wolff, 2013; Wolff et al., 2013, 2014).

Species in this group have a very restricted habitat tolerance. They occur only in humid primary forests, being absent in most secondary forests and degraded environments (Guimarães, 1977). A potential role as bioindicator has already been suggested (Gadelha et al., 2009; Cabrini et al., 2013). Despite the fact that adults feed on both decomposing animal matter and fermented fruit substrates, little is known about their biology, especially in the larval stages. In fact, larvae are

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Table 1
Currently known species of Mesembrinellidae. Distribution information comprises a compilation of data from Guimarães (1977), Bonatto and Marinoni (2005), Wolff (2013), Wolff et al. (2013, 2014) and one unpublished work (Bonatto, 2001). Some new data from Colombia are also presented.

Subfamily classification (*sensu* Guimarães, 1977)	Species	Distribution
Laneellinae	*Laneella nigripes* Guimarães, 1977	Brazil (SE); Paraguay
	Laneella perisi (Mariluis, 1987)	Costa Rica; Colombia; Ecuador; Brazil (NW)
Souzalopesiellinae	*Souzalopesiella facialis* (Aldrich, 1922)	Central America (except Mexico); Venezuela; Trinidad
Mesembrinellinae	*Mesembrinella bellardiana* Aldrich, 1922	Argentina; Paraguay; Brazil (all regions); Peru; Bolivia; French Guayana; Suriname; Venezuela; Colombia; Ecuador
	Mesembrinella peregrina Aldrich, 1922	Brazil (SE)
	Mesembrinella bicolor (Fabricius, 1805)	Central and South America (except Chile and Argentina)
	Mesembrinella abaca (Hall, 1948)	Brazil (SE and NE); Panama; Costa Rica
	Mesembrinella batesi Aldrich, 1922	Brazil (except southern states); Peru; Ecuador; Colombia; Venezuela; Trinidad; French Guyana
	Mesembrinella brunnipes Surcouf, 1919	Bolivia; Peru
	Mesembrinella townsendi Guimarães, 1977	Peru; Colombia; Brazil (NW)
	Mesembrinella apollinaris Séguy, 1925	Colombia
	Mesembrinella currani Guimarães, 1977	Bolivia; Colombia; Brazil (NW)
	Mesembrinella patriciae Wolff, 2013	Colombia
	Mesembrinella umbrosa Aldrich, 1922	Colombia; Panama; Costa Rica
	Mesembrinella pictipennis Aldrich, 1922	Bolivia; Colombia; Costa Rica
	Mesembrinella semihyalina Mello, 1967	Brazil (NE and SE)
	Mesembrinella xanthorrina (Bigot, 1887)*	Bolivia; Peru; Colombia; Panama; Costa Rica; Mexico
	Mesembrinella flavicrura Aldrich, 1925*	Panama; Costa Rica
	Albuquerquea latifrons Mello, 1967	Brazil (SE)
	Henriquella spicata (Aldrich, 1925)	Costa Rica
	Eumesembrinella quadrilineata (Fabricius, 1805)	Brazil (NW); Bolivia; Peru; Ecuador; Colombia; Venezuela; Guyana
	Eumesembrinella benoisti (Séguy, 1925)	Brazil (NW); Venezuela; Guyana; French Guyana
	Eumesembrinella randa (Walker, 1849)	Brazil (NW); Bolivia; Peru; Colombia; Venezuela; French Guyana
	Eumesembrinella cyaneicincta (Surcouf, 1919)	Brazil (SE and NE); Colombia
	Thompsoniella andina Wolff et al., 2014	Colombia
	Thompsoniella anomala Guimarães, 1977	Bolivia; Ecuador; Venezuela
	Giovanella bolivar Bonatto and Marinoni, 2005	Venezuela
	Giovanella carvalhoi Wolff et al., 2013	Colombia
	Huascaromusca semiflava (Aldrich, 1925)	Costa Rica
	Huascaromusca bequaerti (Séguy, 1925)	Peru
	Huascaromusca purpurata (Aldrich, 1922)	Brazil (SE); Peru; Ecuador
	Huascaromusca aeneiventris (Wiedemann, 1830)	Brazil (SE); Ecuador; Colombia; Panama; Costa Rica
	Huascaromusca vogelsangi Mello, 1967	Venezuela; Colombia
	Huascaromusca uniseta (Aldrich, 1925)	Costa Rica
	Huascaromusca decrepita (Séguy, 1925)	Colombia; Venezuela; Mexico
	Huascaromusca lara Bonatto and Marinoni, 2005	Venezuela

*These species were reallocated in the genus *Huascaromusca* by Bonatto (2001) due to the presence of a row of discal setae, although weakly developed, in the abdominal T_5. The proposed combinations seem correct, but because they remain unpublished, the current valid names place these species in the genus *Mesembrinella*.

retained in the female abdomen for extended periods of "gestation", nourished by secretions of the female genital tract (Guimarães, 1977), and a single individual develops and is released into the environment (obligate pseudo-placental unilarviparity; Meier et al., 1999). Attempts to raise larvae outside the female body were unsuccessful except for some larvae of *Laneella nigripes*, which survived through the pupal stage in media containing different animal substances (Guimarães, 1977). This kind of larviparity is singular among Diptera, with equivalents found only in the Hippoboscoidea (Diptera: Calyptrate), even though structures involved in larval nourishment are different (Guimarães, 1977). The group, thus, may represent a very important model for studies of the evolution of complex reproductive biology in Diptera. In this context, a robust and reliable phylogeny is of key importance.

The position of the Mesembrinellidae in the Oestroidea has been contentious. Historically, the group has been treated as a subfamily of Calliphoridae (e.g. Mello, 1967; James, 1970; Hennig, 1973; Pape, 1992; Rognes, 1997), but different authors noted and highlighted the aberrant nature of the Mesembrinellinae within Calliphoridae (Hall, 1948; Crosskey, 1965).

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Guimarães (1977) was the first to formally propose the group as a monophyletic lineage separated from the Calliphoridae, giving family status to the group. This was based on the following morphological and reproductive features of the species in the clade: (1) metathoracic spiracle with a single, large, reniform lappet, with a dorsal opening (i.e. the lappet is discontinuous, being absent from the dorsal spiracular rim); (2) spermathecae with the shape of long, sclerotized tubes; (3) female with all abdominal sternites oval to nearly square-shaped (i.e. not elongated, with the last two sternites not forming a telescopic ovipositor); (4) wing vein M with an evenly curved bend; (5) macrolarviparous habit (adenotrophic viviparity) (Guimarães, 1977). This proposition was supported more recently, although sometimes circumstantially, by phylogenetic analyses based on morphological (Rognes, 1997) and molecular data (Kutty et al., 2010; Marinho et al., 2012; Singh and Wells, 2013; Winkler et al., 2015). There is still controversy, however, on the position of the family within the Oestroidea.

Relationships among the subgroups of Mesembrinellidae are also controversial, mostly due to lack of resolution and weak support found in previous studies. Guimarães (1977), in his description and revision of Mesembrinellidae, made some comments regarding the relationships among species and genera, establishing subfamilies and tribes (Table 1), although without a formal phylogenetic analysis. Toma and Carvalho (1995) were the first to formally conduct a cladistic study in the family, with emphasis on the genus *Eumesembrinella*. The study, using calliphorid species as outgroups, recovered the genus *Laneella* as sister to the remaining Mesembrinellidae, and *Souzalopesiella* as the sister group of all mesembrinellids except *Laneella*. This in large part corroborates Guimarães' (1977) proposal. The genera *Eumesembrinella*, *Huascaromusca* and *Thompsoniella* were recovered in a clade by Toma and Carvalho (1995), whereas *Mesembrinella* and *Albuquerquea* appeared in a large polytomy, the former being paraphyletic (Fig. S1a).

A new study of the family was conducted by Bonatto (2001), including a cladistic analysis. The study comprised a revision of the family, with the description of two new genera (*Henriquela* and *Giovanella*) and three new species (*Giovanella bolivar*, *Henriquela spicata* and *Huascaromusca lara*) (Bonatto and Marinoni, 2005). Moreover, it included a proposition to transfer two species from *Mesembrinella* (*M. xanthorrina* and *M. flavicrura*) to *Huascaromusca* (unpublished). Bonatto (2001) also performed a phylogenetic analysis, having Ameniinae as the immediate outgroup —following a previous study made by Rognes (1997) proposing these two groups as sister taxa. Most of the clades found by Toma and Carvalho (1995) were recovered. *Laneella* appeared as sister to the remaining Mesembrinellidae and *Souzalopesiella* was recovered as sister to the Mesembrinellidae excluding *Laneella*. The rest of the family was recovered in two clades: (1) *Mesembrinella* + *Albuquerquea*, recovered in a large polytomy with the former genus appearing as polyphyletic; and (2) (*Henriquela*, (*Eumesembrinella*, (*Thompsoniella*, (*Giovanella* + *Huascaromusca*)))), all of these genera appearing as monophyletic (Fig. S1b).

Despite the differences in taxon sampling and choice of outgroups between these two studies, they share a pair of general conclusions: (1) most morphological characters used in the analyses are strongly homoplastic; and (2) some genera, especially *Mesembrinella*, in the way they are presently defined, lack unique, exclusive diagnostic morphological characters and generic boundaries are often blurred and hard to delimit. It is worth noting that the biology of the group, with a remarkable dependence on primary forest habitats, makes the distribution frequently disjointed. This quite certainly can lead to genetic differentiation without morphological distinction, bringing problems to establish species boundaries. In this situation, subspecies differing solely or mostly in colour patterns were recognized by Guimarães (1977) for at least two species, *M. bellardiana* and *E. cyaneicincta*.

We present in this study the first hypothesis for phylogenetic relationships among species and genera of Mesembrinellidae based on molecular data. This provides an alternative test for the relationships proposed so far based on morphological data (Guimarães, 1977; Toma and Carvalho, 1995; Bonatto, 2001). Previous studies were conducted using some few calliphorids as outgroups. This study provides the first phylogenetic analysis testing the monophyly of the group with a large sample of Oestroidea species. The taxon sampling of outgroups, however, is not enough to properly address the issue of the position of the family in the system. The sampling in this study included specimens from different localities and regions and allows investigations into molecular differentiation of populations and insights into species boundaries.

Materials and methods

Taxonomic sampling, DNA extraction and PCR amplification

A total of 80 terminal taxa were sampled for the phylogenetic analysis (Table 2). The information on some of the species used comes from a previous study on Oestroidea relationships (Marinho et al., 2012). The taxonomic sampling comprised 28 species of the superfamilies Hippoboscoidea, Muscoidea and Oestroidea (used as outgroups) and 52 specimens of Mesembrinellidae (22 species in 5 genera).

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Table 2
Taxon sampling of Mesembrinellidae and outgroup species used in this study. Classifications provided are based on McAlpine (1989) for outgroup taxa, Rognes (1997) for Calliphoridae subfamilies and Guimarães (1977) for Mesembrinellidae.

						Molecular Markers (GB accession number)				
Superfamilies	Families	Subfamilies	Species	Locality	Sex	*COI*	*COII*	*16S*	*ITS2*	*28S*
Hippoboscoidea	Glossinidae	–	*Glossina morsitans*	–	–	JQ246706	KR820741	JQ246760	–	JQ246656
	Hippoboscidae	Ornithomyinae	*Ornithoctona erythrocephala*	–	–	JQ246707	KR820742	JQ246761	–	JQ246657
Muscoidea	Fanniidae	–	*Fannia* sp.	–	–	JQ246705	KR820743	JQ246759	–	JQ246655
	Muscidae	Cyrtoneurininae	*Cyrtoneuropsis maculipennis*	–	–	–	KR820744	JQ246758	–	JQ246654
		Muscinae	*Musca domestica*	–	–	JQ246703	KR820745	JQ246756	–	JQ246652
Oestroidea	Oestridae	Cuterebrinae	*Dermatobia hominis*	–	–	JQ246701	KR820746	JQ246754	–	JQ246650
			Cuterebra sp.	–	–	JQ246700	KR820747	JQ246753	–	JQ246649
		Oestrinae	*Oestrus ovis*	–	–	KR820703	KR820748	KR820848	–	KR820885
	Tachinidae	Dexiinae	*Prophorostoma pulchra*	–	–	JQ246699	KR820749	JQ246751	–	JQ246647
		Exoristinae	*Chetogena* sp.	–	–	JQ246697	KR820750	JQ246749	–	JQ246645
	Sarcophagidae	Sarcophaginae	*Sarcophaga bullata*	–	–	JQ246696	KR820751	JQ246748	–	JQ246644
			Peckia ingens	–	–	–	KR820752	JQ246747	–	JQ246643
	Rhiniidae	–	*Rhinia* sp.	–	–	JQ246692	KR820753	JQ246743	–	JQ246640
			Cosmina fuscipennis	–	–	JQ246691	KR820754	JQ246742	–	JQ246639
	Calliphoridae	Polleniinae	*Pollenia rudis*	–	–	–	KR820755	KR820849	–	KR820886
		Bengaliinae	*Bengalia peuhi*	–	–	JQ246685	KR820756	JQ246734	–	JQ246631
		Auchmeromyinae	*Pachychoeromyia praegrandis*	–	–	JQ246683	KR820757	JQ246732	–	JQ246629
			Cordylobia anthropophaga	–	–	JQ246681	KR820758	JQ246730	–	JQ246627
		Toxotarsinae	*Sarconesia chlorogaster*	–	–	JQ246674	KR820759	JQ246723	–	JQ246619
		Luciliinae	*Lucilia eximia*	–	–	JQ246678	KR820760	JQ246727	–	JQ246623
			Lucilia cuprina	–	–	JQ246677	KR820761	JQ246726	–	JQ246622
		Calliphorinae	*Calliphora croceipalpis*	–	–	JQ246671	KR820762	JQ246720	–	JQ246616
			Calliphora vicina	–	–	JQ246672	KR820763	JQ246721	–	JQ246617
		Chrysomyinae	*Cochliomyia hominivorax*	–	–	JQ246665	KR820764	JQ246714	–	JQ246610
			Chrysomya megacephala	–	–	JQ246662	KR820765	–	–	JQ246607
			Hemilucilia semidiaphana	–	–	JQ246668	KR820766	JQ246717	–	JQ246613
			Protophormia terraenovae	–	–	JQ246670	KR820767	JQ246719	–	JQ246615
			Chloroprocta idioidea	–	–	JQ246658	KR820768	JQ246708	–	JQ246603
	Mesembrinellidae	Laneellinae	*Laneella perisi*	CA/CO	F	KR820704	–	KR820850	KR820806	KR820887
			Laneella nigripes ind. 1	SP/BR	F	KR820705	–	KR820851	KR820807	KR820888
			Laneella nigripes ind. 2	SP/BR	F	KR820706	KR820769	KR820852	KR820808	KR820889
			Laneella nigripes ind. 3	SP/BR	M	–	–	–	KR820809	KR820890
		Mesembrinellinae	*Mesembrinella apollinaris*	CA/CO	F	KR820707	KR820770	KR820853	KR820810	KR820891
			Mesembrinella batesi ind. 1	CA/CO	M	KR820708	KR820771	KR820854	KR820811	KR820892
			Mesembrinella batesi ind. 2	AM/BR	M	KR820709	KR820772	KR820855	KR820812	KR820893
			Mesembrinella batesi ind. 3	AM/BR	M	KR820710	KR820773	KR820856	KR820813	KR820894
			Mesembrinella batesi ind. 4	AM/BR	F	KR820711	KR820774	KR820857	KR820814	KR820895
			Mesembrinella batesi ind. 5*,†,‡	AM/BR	N/A	–	KR820784	JQ246739	EU076456	JQ246636
			Mesembrinella bellardiana (SE) ind. 1	MG/BR	M	KR820712	KR820775	KR820858	KR820815	KR820896
			Mesembrinella bellardiana (SE) ind. 2	MG/BR	F	KR820713	KR820776	KR820859	KR820816	KR820897
			Mesembrinella bellardiana (SE) ind. 3	SP/BR	M	KR820714	KR820777	KR820860	KR820817	KR820898
			Mesembrinella bellardiana (NW) ind. 1†	AM/BR	N/A	JQ246688	KR820778	JQ246738	JQ246586	JQ246635

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Table 2
(Continued)

Superfamilies	Families	Subfamilies	Species	Locality	Sex	Molecular Markers (GB accession number)				
						COI	*COII*	*16S*	*ITS2*	*28S*
			Mesembrinella bellardiana (NW) ind. 2†	AM/BR	N/A	–	–	JQ246737	EU076455	JQ246634
			Mesembrinella bellardiana (NW) ind. 3	CA/CO	F	KR820715	–	KR820861	KR820818	KR820899
			Mesembrinella bicolor ind. 1†	PA/BR	F	JQ246689	–	JQ246740	JQ246587	JQ246637
			Mesembrinella bicolor ind. 2	CA/CO	F	KR820716	KR820779	KR820862	KR820819	KR820900
			Mesembrinella bicolor ind. 3*,†,§	SP/BR	N/A	JQ246690	–	JQ246741	EF560188	JQ246638
			Mesembrinella currani	CA/CO	F	KR820717	KR820780	KR820863	KR820820	KR820901
			Mesembrinella patriciae	QU/CO	F	KR820718	KR820781	KR820864	KR820821	KR820902
			Mesembrinella peregrina ind. 1	SP/BR	M	–	KR820782	–	KR820822	KR820903
			Mesembrinella peregrina ind. 2†	SP/BR	N/A	KR820720	KR820785	KR820866	–	–
			Mesembrinella pictipennis	CA/CO	F	KR820719	KR820783	KR820865	KR820823	KR820904
			Mesembrinella townsendi ind. 1	AN/CO	M	KR820723	KR820788	–	KR820826	KR820907
			Mesembrinella townsendi ind. 2	AN/CO	F	KR820724	KR820789	KR820869	KR820827	KR820908
			Mesembrinella townsendi ind. 3	CA/CO	M	KR820721	KR820786	KR820867	KR820824	KR820905
			Mesembrinella townsendi ind. 4	CA/CO	F	KR820722	KR820787	KR820868	KR820825	KR820906
			Eumesembrinella benoisti ind. 1	AM/BR	N/A	JQ246686	KR820790	JQ246735	JQ246584	JQ246632
			Eumesembrinella benoisti ind. 2	AM/BR	M	KR820725	KR820791	KR820870	KR820828	KR820909
			Eumesembrinella benoisti ind. 3	AM/BR	F	KR820726	KR820792	KR820871	KR820829	KR820910
			Eumesembrinella cyaneicincta (SE) ind. 1	RJ/BR	F	KR820727	KR820793	KR820872	KR820830	KR820911
			Eumesembrinella cyaneicincta (SE) ind. 2	RJ/BR	F	–	–	–	–	KR820912
			Eumesembrinella cyaneicincta (NW) ind. 1	CA/CO	M	KR820728	KR820794	KR820873	KR820831	KR820913
			Eumesembrinella cyaneicincta (NW) ind. 2	CA/CO	F	KR820729	KR820795	–	KR820832	KR820914
			Eumesembrinella quadrilineata ind. 1	PA/BR	F	JQ246687	KR820796	JQ246736	JQ246585	JQ246633
			Eumesembrinella quadrilineata ind. 2	AM/BR	M	KR820730	KR820797	KR820874	KR820833	KR820915
			Eumesembrinella quadrilineata ind. 3	AM/BR	F	KR820731	KR820798	KR820875	KR820834	KR820916
			Eumesembrinella randa ind. 1	AM/BR	M	KR820732	KR820799	KR820876	KR820835	KR820917
			Eumesembrinella randa ind. 2	AM/BR	F	KR820733	KR820800	KR820877	KR820836	KR820918
			Eumesembrinella randa ind. 3	AM/BR	F	KR820734	KR820801	KR820878	KR820837	KR820919
			Eumesembrinella randa ind. 4	AM/BR	F	KR820735	KR820802	KR820879	KR820838	KR820920
			Giovanella carvalhoi	CA/CO	M	KR820736	KR820803	KR820880	KR820839	KR820921
			Huascaromusca sp. 1 (*obscura*) ind. 1¶	AN/CO	F	KR820737	KR820804	KR820882	KR820842	KR820923
			Huascaromusca sp. 1 (*obscura*) ind. 2**	CO	N/A	KC568269	–	–	–	–
			Huascaromusca aeneiventris ind. 1	SP/BR	M	–	–	–	KR820840	KR820922
			Huascaromusca aeneiventris ind. 2	SP/BR	M	–	–	KR820881	KR820841	–
			Huascaromusca aeneiventris ind. 3	SP/BR	F	–	–	KR820884	KR820844	–
			Huascaromusca purpurata	SP/BR	F	KR820738	–	KR820883	KR820843	KR820924

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Table 2
(*Continued*)

Superfamilies	Families	Subfamilies	Species	Locality	Sex	Molecular Markers (GB accession number)				
						COI	*COII*	*16S*	*ITS2*	*28S*
			Huascaromusca sp. 2¶	CA/CO	F	KR820739	–	–	KR820845	KR820925
			Huascaromusca vogelsangi ind. 1	CA/CO	M	–	–	–	KR820846	KR820926
			Huascaromusca vogelsangi ind. 2	CA/CO	F	KR820740	KR820805	–	KR820847	KR820927

N/A, data not available. M & F, males and females, respectively. (SE) & (NW), south-east and north-west populations from South America.
*Mesembrinellidae species with incorrect identification provided in a previous study (Marinho et al., 2012). Localities: AM, Amazonas; AN, Antioquia; CA, Caquetá; MG, Minas Gerais; PA, Pará; QU, Quindío; RJ, Rio de Janeiro; SP, São Paulo; BR, Brazil; CO, Colombia.
†Mesembrinellidae specimens for which there is no voucher.
‡Specimen initially identified as *M. bellardiana*, but with molecular identification closer to *M. batesi*.
§Specimen initially identified as *M. peregrina*, but with molecular identification closer to *M. bicolor*.
¶New species of *Huascaromusca*, whose description will be published elsewhere. For *Huascaromusca* sp. 1, a provisional name, *H. obscura*, is provided.
**Sequence extracted from GenBank (Solano et al., 2013). A reassessment of the material indicated it is actually an *Huascaromusca* species, the same as *Huascaromusca* sp. 1.

DNA extractions were carried out with the Illustra Tissue and Cells GenomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK), using 2–3 legs per individual. The specimens were photographed and kept in alcohol in a −20 °C freezer, serving as identification vouchers if necessary (Fig. 1). Five genomic regions were PCR-amplified and used as molecular markers: (1) the whole region of the Second Internal Transcribed Spacer (*ITS2*) and (2) the 5′ region of the large ribosomal DNA subunit (*28S* rDNA), both from the nuclear ribosomal DNA cluster; (3) the 5′ region of the first subunit of the cytochrome oxidase gene (*COI*); (4) the whole coding region of the second subunit of the cytochrome oxidase gene (*COII*); and (5) the 3′ end of the large rDNA subunit (*16S* rDNA), the last three from the mitochondrial genome. The *ITS2*, *28S*, *COI* and *16S* regions were amplified using the primers and reaction conditions described in Marinho et al. (2012). The *COII* gene was amplified with the same reaction conditions as the *COI* gene, using the primers TL2-J3034 (5′-AATATGGCAGATTAGTGCA-3′) and TK-N3785 (5′-GTTTAAGAGACCAGTACTTG-3′) (Simon et al., 1994). All amplified fragments were purified and sequenced directly from the PCR products, except the *ITS2* region, which was cloned, as described in Marinho et al. (2012).

Secondary structure prediction and modelling

Secondary structure modelling for the *ITS2*, *28S* and *16S* regions was conducted using a similar approach as described by Marinho et al. (2012). Briefly, regions *28S* and *16S* were modelled based on the described secondary structures for these regions in *Drosophila melanogaster* (available at the Comparative RNA database, CRWeb; Cannone et al., 2002). Some more variable helices were modelled *de novo* using the mfold software (Zuker, 2003). Nomenclature for the domains and helices follows Gillespie et al. (2006).

For the *ITS2* region, we used a combined approach of *de novo* and homology-based modelling, comprising: (1) *in silico* prediction based on thermodynamic parameters with the mfold software (Zuker, 2003); (2) comparison with previously published structures for *D. melanogaster* (Young and Coleman, 2004) and some species of the Calliphoridae and Mesembrinellidae families (Marinho et al., 2011, 2012, 2013); and (3) comparison among the structures predicted for all species and the establishment of a common folding pattern.

All predicted structures were drawn using the VARNA 3.91 software (Darty et al., 2009).

Sequence alignment and congruence test

Sequence alignment was initially conducted with the software MAFFT v7.149 (Katoh et al., 2002; Katoh

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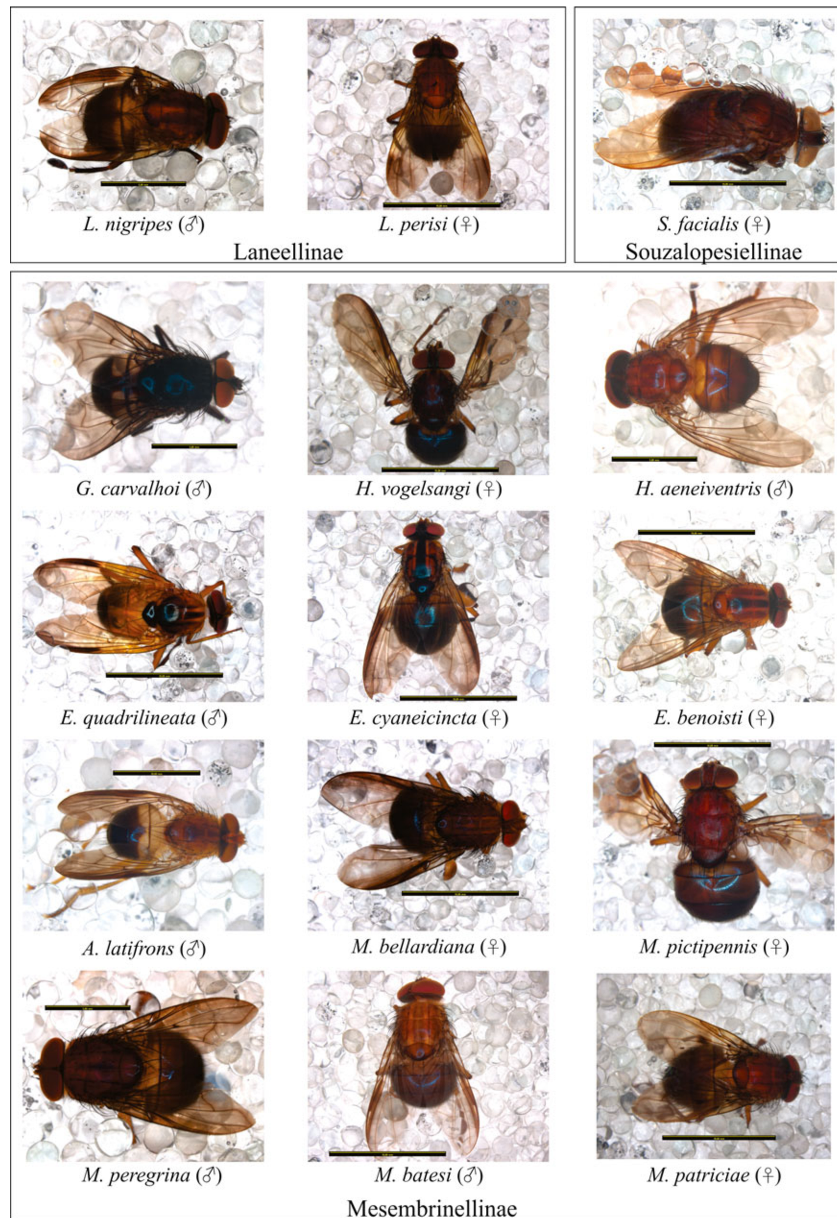

Fig. 1. Some of the Mesembrinellidae species sampled for this study. Specimens of *S. facialis* and *A. latifrons* were obtained, but no markers could be amplified, probably due to inadequate preservation. Subfamily classification follows Guimarães (1977). [Colour figure can be viewed at wileyonlinelibrary.com].

and Standley, 2013). Protein coding regions (*COI* and *COII*) were aligned using the G-INS-i module, whereas the noncoding regions (*ITS2*, *28S* and *16S*) used the Q-INS-i module.

The resulting alignments were analysed with the "Alignment Explorer" tool of the software MEGA 6 (Tamura et al., 2013). Aligned protein coding regions were then translated into amino acid sequences, inspected for out-of-frame indels and premature stop codons, re-aligned using the plug-in of the MUSCLE software (Edgar, 2004) and finally back-translated in nucleotide sequences. For the alignments of the noncoding regions (*ITS2*, *28S* and *16S*), secondary structure masks (dot-bracket format) were added to the respective sequences and inspected for regions with ambiguously aligned sites (loop regions). These regions were then locally aligned using the MUSCLE plug-in and some final manual adjustments were conducted. For the *ITS2* region, only Mesembrinellidae sequences were used, in order to minimize potential erroneously aligned regions and, therefore, mistaken nonhomologies for the sequences. For the same reason, domain IV, which is almost completely comprised of small sequence repeats (As, Ts and ATs), were also excluded from the

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analyses. The final aligned datasets comprised 755 bp for *COI*, 688 bp for *COII*, 680 bp for *16S* rRNA, 587 bp for *ITS2* and 1365 bp for *28S* rRNA regions (4075 nucleotides in the final concatenated dataset; see “Matrix S1”, Supplementary Documentation).

Individual alignments were tested for congruence among partitions (individual genes), for both topology and branch lengths, with the software Concaterpillar 1.4 (Leigh et al., 2008), which uses the software RAxML-VI-HPC (Stamatakis, 2006) for likelihood estimations.

Model selection and Phylogenetic analyses

Maximum parsimony (MP) analyses were conducted in the software TnT v1.1 (Goloboff et al., 2008), under the “New Technology Search” option (search at level = 50; initial addseqs = 15; find minimum tree length 10 times). Two analyses were performed, considering gaps as “missing data” or as “fifth character state”. Node supports were assessed by bootstrap (BS) resampling with 1000 replicates, using the same options as in the original search.

Model selection for the maximum-likelihood (ML) and Bayesian inference (BI) analyses was carried out with the program jModelTest v2.1.5 (Darriba et al., 2012). For both methods, different data partition schemes were tested and models were selected individually for each partition. Alternatively, we used the software PartitionFinder v1.1.1 (Lanfear et al., 2012) to find partition schemes with substitution models that best fit the data, using the default parameters of the program and a greedy search algorithm. In both ML and BI analyses, models applied to each partition were considered unlinked, with parameters inferred from distinct distributions [GARLI: linkmodels = 0, subsetspecificrates = 1; MrBayes: prset applyto = (all) ratepr = variable, unlink statefreq = (all) revmat = (all) shape = (all) pinvar = (all)].

ML analyses were conducted in the software GARLI v2.0 (Zwickl, 2006) (five independent searches; 20 000 000 generations; default options for automated stop). Three different partition strategies were used: (1) a minimum partition scheme, considering each gene as a separate partition evolving under its best-fitted model (5 partitions: ML-MinPart); (2) a fully partitioned scheme, adding further partitions for the three codon positions of the protein coding genes (9 partitions: ML-FullPart); and (3) an intermediate partition scheme, defined by the PartitionFinder software, which combined each codon position of the two protein coding genes (*COI* and *COII*) in a single partition (6 partitions: ML-MidPart). A summary of these partition strategies with the models used is shown in Table 3. Node support values were assessed by bootstrap (BS) resampling (1000 replicates for the 5-partition strategy and 100 for the 6- and 9-partition ones) with relaxed search options (genthreshfortopoterm = 10 000).

Table 3
Summary of the partition strategies and substitution models used in the maximum-likelihood (ML) and Bayesian inference (BI) analyses.

	COI			*COII*			*16S*		*ITS2*		*28S*	
Partition Strategy	1st	2nd	3rd	1st	2nd	3rd	ss	ds	ss	ds	ss	ds
ML												
MinPart		GTR + I + G			GTR + I + G		GTR + I + G		GTR + G		GTR + I + G	
MidPart (PartitionFinder)	GTR + I + G [A]	GTR + I + G [B]	HKY + I + G [C]	GTR + I + G [A]	GTR + I + G [B]	HKY + I + G [C]	GTR + I + G [D]		GTR + G [E]		GTR + I + G [F]	
FullPart	GTR + I + G	HKY + I	HKY + G	HKY + G	HKY + I + G	GTR + G	GTR + I + G		GTR + G		GTR + I + G	
BI												
MinPart		GTR + I + G			GTR + I + G		GTR + I + G		GTR + G		GTR + I + G	
MidPart (PartitionFinder)	GTR + I + G [A]	GTR + I + G [B]	HKY + I + G [C]	GTR + I + G [A]	GTR + I + G [B]	HKY + I + G [C]	GTR + I + G [D]	Doublet* (GTR + I + G) [E]	GTR + G [F]	Doublet* (HKY + I + G) [G]	GTR + I + G [D]	Doublet* (HKY + I + G) [G]
FullPart	GTR + I + G	HKY + I	HKY + G	HKY + G	HKY + I + G	GTR + G	GTR + I + G	Doublet (GTR + I + G)	GTR + G	Doublet (GTR + G)	GTR + I + G	Doublet (GTR + I + G)

1st, 2nd & 3rd, codon positions for protein coding regions. ss and ds, single and double-stranded regions (respectively) in the secondary structures of the RNA coding regions. For the MidPart scheme, regions with the same letter inside “[ ]s” were included in the same partition.

*The PartitionFinder software does not implement the doublet model, thus the resulting models given by the software were used to set the substitution models underlying the doublet model in MrBayes.

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BI analyses were conducted using MrBayes v3.2.6 (Ronquist et al., 2012), available at the CIPRES Science Gateway v3.3 (Miller et al., 2010). As for the ML analyses, three distinct partition strategies were used: (1) each gene region being considered as a single partition (5 partitions: BI-MinPart); (2) each gene region as a single partition, further divided by codon position in the protein coding genes and secondary structure conformation (single- or double-stranded) in the RNA coding regions (12 partitions: BI-FullPart); and (3) a combination of some of the partitions considered in scheme (2), as defined by the PartitionFinder software (7 partitions: BI-MidPart) (Table 3). The consensus secondary structures for the latter two, necessary for the implementation of the nonindependent site evolution model ("Doublet"), were inferred with the software "secondarystructconsensus" of the PHASE 2.0 package (Gowri-Shankar and Jow, 2006). All partition strategies were run in duplicates (two independent runs to check for consistency) for 30 000 000 generations, with two sets of 6 chains, sample frequency = 1000 and burn-in set to 33% (or higher, if necessary) after checking the summary statistics for convergence. Node supports for all BI analyses were assessed by analysing the *a posteriori* probabilities (PP) in the 50% extended majority-rule consensus tree (option "sumt contype = allcompat" in MrBayes).

The different partition schemes in the BI analyses were then compared for performance in (1) the MCMC run (number of generations to convergence); (2) topology estimation (number of trees included in the 95% and 99% confidence intervals; tree length; average node support; and topological differences among strategies, the latter evaluated using symmetric distances calculated in the TreeDist software; Felsenstein, 2005); and (3) parameter estimation process (Effective Sample Size, ESS) and overall performance (Marginal lnL).

For the overall performance comparison, we used the Bayes Factor statistics, with interpretation for the results following the table provided by Kass and Raftery (1995). The Marginal lnL for each partition scheme (combination of models), necessary for the Bayes Factor calculation, were estimated using the stepping-stone sampling method (Fan et al., 2011; Xie et al., 2011), which was based on a stepping-stone ("ss") run of 60 000 000 generations in MrBayes 3.2.6 (at the CIPRES v3.3. Science Gateway) for each partition strategy.

Results

Secondary structure prediction

Secondary structure models for the *16S*, *28S* and *ITS2* regions are shown in Figs S2–S4. For the regions *16S* and *28S*, the nomenclature adopted was based on the proposal by Gillespie et al. (2006). This nomenclature differs from the one used in a previous analysis (Marinho et al., 2012), which was based on an older publication by Buckley et al. (2000). For the region *ITS2*, the nomenclature adopted is based on the works of Young and Coleman (2004) and Marinho et al. (2011, 2012, 2013).

Predicted structures for the *16S* and *28S* rRNAs are very similar to the ones proposed for the genus *Drosophila*, available at the Comparative RNA Web (CRWeb) (Cannone et al., 2002). Some helices were, however, variable in their conformation and their structures were predicted *de novo* using the mfold software (Zuker, 2003). For the *16S* rRNA, these helices included H1835, H2077 and H2347, equivalent to helices H68, H75 and H84 in the nomenclature of Buckley et al. (2000), respectively. The latter two were more variable and some of the predicted conformations are shown in Fig. S2 (supplementary documentation).

For region *28S*, a considerable size variation was observed in the helices comprising both expansion domains 2 (helices D2-2 and D2-3) and 3 (D3) in Oestridae species. In domain D3, two helices were found consistently in all species analysed, corresponding to helices D3-2 and D3-3 of *Apis mellifera* (Gillespie et al., 2006). Nevertheless, in all predicted structures analysed, no homologue of helix D3-1 described by Gillespie et al. (2006) was found. This helix is also absent in the structure described for *D. melanogaster* (Cannone et al., 2002). There is, however, a helix in the region corresponding to the D3-1 in Oestridae and in some of the mesembrinellid species analysed (*M. bellardiana*, *M. apollinaris*, *M. peregrina*, *G. carvalhoi* and all *Eumesembrinella*), but this helix does not possess any primary sequence or secondary structural features suggesting homology to the helix of *A. mellifera*. In fact, these helices were actually composed of many As and Ts and probably evolved by varying the number of repeats of these short polymeric elements without functional restraints.

For the region *ITS2*, domains I and II, as well as the proximal and end portions of domain III, were very conserved and easily modelled based on the structures previously described for calliphorids (Marinho et al., 2012, 2013). The mid-portion of domain III, however, significantly differs from the structures presented for Calliphoridae, in which two lateral ramifications with very conserved primary sequence motives emerge from a medial junction. In Mesembrinellidae, the mid portion of domain III may fold in three distinct structural conformations, presenting somewhat similar free energy values (ΔGs). Two of these structures are branched, one with a single branch (on the 5′ side; a more stable structure) and the other with two

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branches (on both 5′ and 3′ sides), whereas the third structural conformation presents no ramifications. Because, in general, the differences in ΔGs values among these structures are small, it is possible that a dynamic conformation pattern exists in this portion of the molecule, with different structures present in solution (at least when only thermodynamic parameters are taken into account), such as described for *M. domestica* (Marinho et al., 2013). Thus, even considering that the structure with a single branch at the 5′ side was the most stable structure (smallest ΔG), this region was left unpaired in the consensus secondary structure used in the BI phylogenetic analyses.

The most variable domain IV was modelled individually for each species, because no common folding pattern was observed. In fact, some species even lack a helix in the corresponding region for this domain (e.g. *M. bicolor*), whereas others present two helices [e.g., *M. bellardiana* (SE)] or a very large, branched helix in this region (*M. peregrina*). Regarding primary sequence composition, domain IV in most species seems to be composed mainly of variable-sized polymeric repeats of As and Ts, evolving without significant functional and structural constrains. Because this kind of pattern hampers the process of establishing positional homology, domain IV was excluded in the subsequent phylogenetic analyses.

Preliminary analyses: congruence test

Results of the test of congruence among partitions showed that all five genes can be concatenated in a single data matrix (tests for topological congruence: $p_{ITS2\text{-}28S} = 0.1259$; $p_{COII\text{-}16S} = 0.5585$; $p_{16S\text{-}COII\text{-}ITS2\text{-}28S} = 0.2075$; $p_{16S\text{-}COII\text{-}ITS2\text{-}28S\text{-}COI} = 0.1404$), but that parameters estimation and optimization should be performed independently (all tests for branch length congruence $P < 0.05$). Based on these results, the simplest model partition strategy used in the phylogenetic analyses comprised each gene evolving under its best-fitted substitution model.

Phylogenetic analyses

Inferred trees among different methods (MP, ML and BI) and partition schemes (MinPart, MidPart and FullPart in the ML and BI analyses) were largely concordant, with some exceptions. Among the Mesembrinellidae, these exceptions comprised (1) the position of *M. peregrina*, with two competing hypotheses recovered by different methods and partition schemes within the same method, and (2) the relationships inside the clade (*Huascaromusca* + *Giovanella*), with all but the MP analysis with gaps considered as fifth character state recovering the same topology. Among outgroup taxa, inferred relationships were almost completely polytomic in both MP analyses and variable among the different partitioning strategies in the ML and BI analyses. In these latter two, the "wandering" behaviour of the long-branched taxon *Oestrus ovis* accounts for most of this variability, because it caused some distortions in the relationships among nearby taxa in the variable positions in which it was recovered.

Despite of this, two main results are very consistent: (1) none of the reconstructions show the mesembrinellids as paraphyletic in relation to any other group of oestroids; and (2) the mesembrinellids never grouped with the core calliphorids. A summary of the results found in all analyses is shown in Figs 2 and S5 (Supplementary documentation).

For a more detailed analysis of the inferred relationships, the Mesembrinellidae was recovered, in all trees, as a monophyletic group inside Oestroidea with high support (MP BS = 97/98; ML BS = 99/100/100; BI PP = 1.00/1.00/1.00), in almost all analyses sister to a clade composed of (Sarcophagidae + *Pollenia rudis*), but with low support.

Relationships within the Mesembrinellidae, with few exceptions, agree among all inference methods and partitioning strategies used, and node supports in this part of the tree are in general high. A clade composed of (*Laneella* + *M. patriciae*) was recovered in all trees and has high support (MP BP = 94/97; ML BP = 100/97/100; BI PP = 1.00/1.00/1.00). This clade is the sister group of all remaining species in the family. The species in the other clade gather into two main clades. One comprises a paraphyletic *Mesembrinella* in relation to *Eumesembrinella*; the other comprises a paraphyletic *Huascaromusca* in relation to *Giovanella*.

For the (*Huascaromusca* + *Giovanella*) clade, all analyses except the MP with gaps as fifth state recovered the same relationships, namely, a clade composed of the Brazilian species nested inside a Colombian grade of species, where *Giovanella* also fits. The strict consensus tree of the MP analysis with gaps as fifth state, however, recovered a clade with the Brazilian and Colombian species of *Huascaromusca*, the latter also including *Giovanella*, as sister groups.

In the *Mesembrinella* + *Eumesembrinella* clade, the ML and BI analyses with more complex partitioning strategies (MidPart and FullPart) recovered *M. peregrina* as sister of a clade including a monophyletic *Eumesembrinella* plus *M. bicolor* and the remaining *Mesembrinella* (excluding *M. patriciae*). Both MP consensus trees and the ML and BI trees inferred with the least complex scheme (MinPart) differed slightly, basically recovering *M. peregrina* as sister of *Eumesembrinella*, a clade to which *M. bicolor* also belongs. The other major *Mesembrinella* clade consistently separates *M. bellardiana* from a clade including (*M. currani* + *M. pictipennis*) and (*M. townsendi* + (*M. apollinaris* + *M. batesi*)) (MP BS = 84/85; ML BS = 100/

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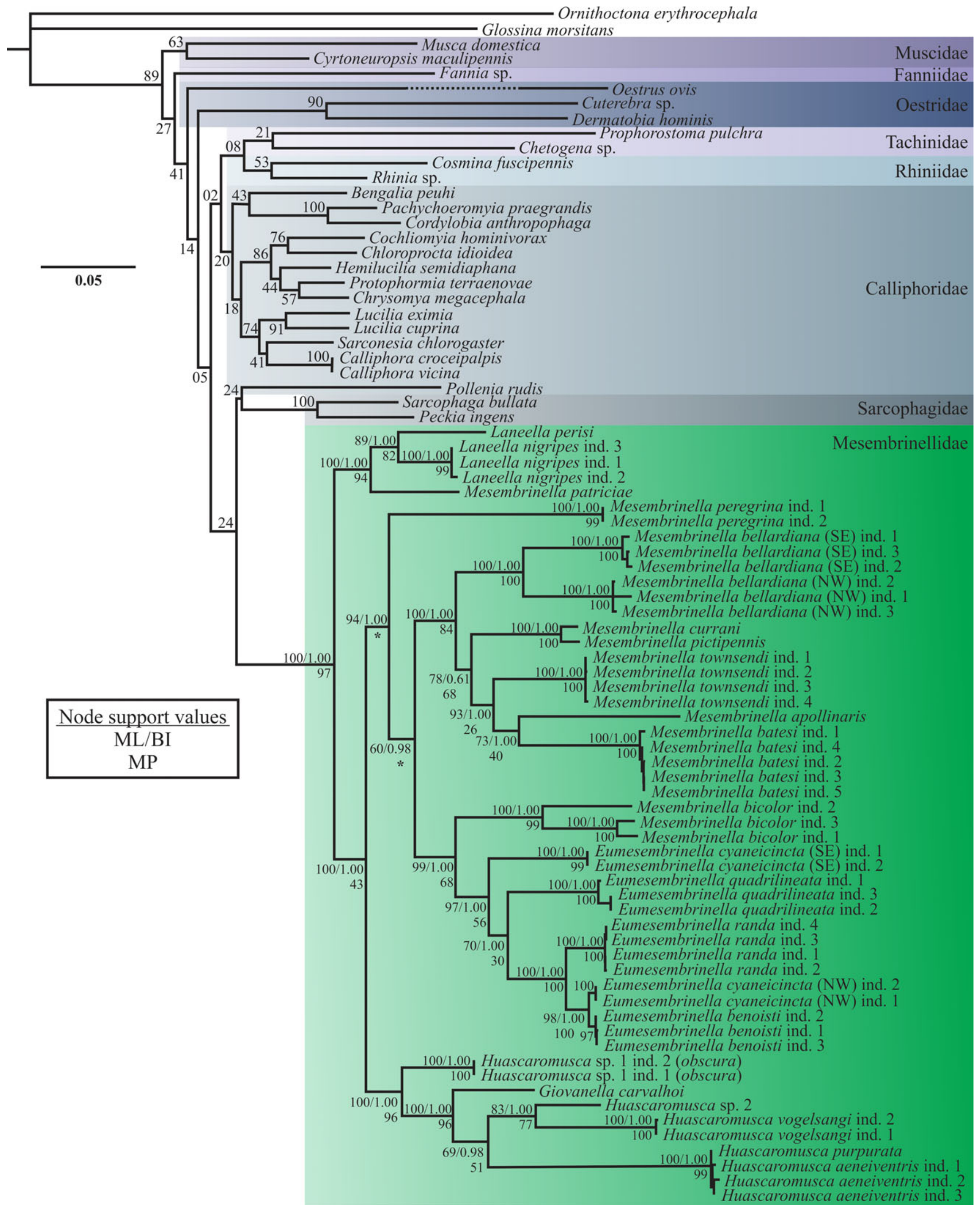

Fig. 2. Maximum-likelihood tree inferred using the FullPart partition strategy (see Table 3 for details). Bootstrap support values are shown next to the respective nodes. For relationships inside the Mesembrinellidae clade, Maximum-Parsimony (bootstrap - "gaps as missing" analysis) and Bayesian Inference (posterior probabilities - FullPart analysis) node support values are also given, following the legend provided in the left side of the figure. * = in the MP analysis, *M. peregrina* was recovered as the sister-taxon of (*M. bicolor* + *Eumesembrinella*). [Colour figure can be viewed at wileyonlinelibrary.com].

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Table 4
Symmetric distances among topologies inferred under different partition schemes in the BI analyses. (A) and (B) refer to duplicated runs of the same partition strategy.

	MinPart (A)	MinPart (B)	MidPart (A)	MidPart (B)	FullPart (A)	FullPart (B)
MinPart (A)	–					
MinPart (B)	0	–				
MidPart (A)	34	0	–			
MidPart (B)	34	34	0	–		
FullPart (A)	42	42	32	0	–	
FullPart (B)	42	42	32	32	0	–

100/100; BI PP = 1.00/1.00/1.00). For *M. bellardiana*, the two populations (SE and NW) were recovered in distinct clades (MP BS = 100/100; ML BS = 100/100/100; BI PP = 1.00/1.00/1.00), separated by deep branches. In agreement with this pattern, specimens from the two populations present both very divergent *COI* sequences (genetic distances within populations = 0.8–2.4% and among populations = 14.4–16.3%) and distinct ITS2 sequences and secondary structures (Fig. S4g, h). Thus, these two populations could represent two distinct species.

Nested inside a paraphyletic *Mesembrinella*, the genus *Eumesembrinella* was recovered as monophyletic with high support in almost all trees (MP BS = 56/97; ML BS = 99/98/97; BI PP = 1.00/1.00/1.00). The two sampled populations of *E. cyaneicincta* (SE and NW) were recovered as distinct clades, with the south-east population being sister to the remaining species in the genus, all of them distributed exclusively in north-west South America. In the NW clade of the genus, *E. quadrilineata* was recovered as the sister taxon of a clade comprising (*E. randa* + (*E. cyaneicincta* (NW) + *E. benoisti*)), the latter with high support in all analyses (MP BS = 100/100; ML BS = 100/100/100; BI PP = 1.00/1.00/1.00). In the case of *E. cyaneicincta*, the two sampled populations actually comprise a pair of distinct species.

Bayesian-based comparison and evaluation of partitioning strategies

The use of different partition schemes had influence on the inferred topologies, mostly on the relationships among outgroup taxa (Table 4). The only exception among mesembrinellid terminal taxa are the two alternative positions recovered for *M. peregrina* (Figs 2 and S5). Among outgroup taxa, however, inferred topologies were more unstable, most notably due to the erratic behaviour of *Oestrus ovis* and the local distortions its long branch caused among the nearby taxa. For this taxon, recovered positions were variable among different partition schemes, but consistent between replicates (Table 4), indicating that the recovered affinities are not due to stochastic errors in the inference procedure. The investigation of the relationships among outgroup taxa is not within the scope of the analyses conducted here. Nevertheless, the high instability in the relationships inferred for outgroup terminal taxa may respond for most of the observed differences in the summary information for topology and parameter estimation and MCMC run diagnosis of the analyses under different partition strategies (Table 5).

Increasing partition scheme complexity led to an overall increase in model fit, as shown by the higher Marginal lnL values found in the MidPart and FullPart strategies (Table 5). All comparisons among partitioning strategies performed with Bayes Factor were significant, strongly favouring the FullPart scheme, the most complex one (Table 6). This increase in model-fitting, nevertheless, led to an increase in tree topology uncertainty, as denoted by the higher number of trees included in the 95% / 99% confidence intervals and the decrease, albeit small, in the average PP support values (Table 5). Although these features are likely to have a direct correlation with model complexity, PP support values among the ingroup terminal taxa seemed to be more directly correlated with overall tree length, the intermediate partition scheme used (MidPart strategy) showing the shorter tree with higher average PP. It is worth noting that this pattern does not parallel the one observed in the ML analyses, in which tree length and BS support values are higher in the most complex partition scheme (Table 5).

Highly partitioned models are associated with more complex parameter space and it is usually expected that longer sampling periods during the stationary phase of the MCMC run are required. As the burn-in fractions were normalized among the different partitioning strategies used, the same number of samples was used to generate the summary information provided in Table 5. As expected, ESS values (the effective number of independent samples taken during the MCMC run), although much higher than usually recommended to be considered a good mixed combination of chains and a well sampled posterior

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Table 5
Summary information for the BI runs performed with different partition schemes. (A) and (B) refer to duplicated runs of the same partition strategy. Entries in the "Tree Length" and "Average Support" cells are the estimated mean for these values, with the corresponding variances given between parentheses "()". The stepping-stone analyses for Marginal lnL estimation were not performed in duplicates. Some summary information for the ML analyses with correspondent partition schemes are given for comparison purposes.

	Partition strategies					
	MinPart (A)	MinPart (B)	MidPart (A)	MidPart (B)	FullPart (A)	FullPart (B)
Generations (1 sample / 10^3)	30×10^6	30×10^6	30×10^6	30×10^6	30×10^6	30×10^6
Number of generations to convergence (approx.)	13.16×10^6*	9.385×10^6	4.71×10^6	11.39×10^6*	2.141×10^6	1.646×10^6
Number of trees (95%/99% confidence interval)	31572 / 32916	37624 / 39232	38082 / 39690	35267 / 36755	38171 / 39779	38171 / 39779
Tree Length (TL)	6.170004 (0.067192)	6.163614 (0.066957)	5.162475 (0.048076)	5.161060 (0.047965)	5.699138 (0.072426)	5.698109 (0.074240)
Average Support (PP)						
Outgroup	0.787808 (0.048492)	0.792692 (0.046472)	0.710462 (0.08673)	0.708462 (0.088141)	0.777538 (0.055703)	0.778962 (0.054872)
Mesembrinellidae	0.913902 (0.035612)	0.914039 (0.035786)	0.917294 (0.031525)	0.917196 (0.031554)	0.907157 (0.040201)	0.906588 (0.040545)
Average ESS (overall)	>3000	>3300	>1600	>1600	>1400	>1400
Marginal lnL (stepping-stone)	−34184.3135		−32278.7985		−32173.01	
ML						
Max. lnL	−34526.4224		−33618.1346		−33586.3453	
Tree Length (TL)	7.826		7.854		8.224	
Average Support (BS) for Mesembrinellidae	81.588235 (793.8871)		81.470588 (752.5341)		81.725490 (771.5631)	

ESS, Effective Sample Size.

*Burn-in values for these runs were set in accordance with the convergence time: 44% for the MinPart (A) and 38% for MidPart (B). In all the remaining runs, the number of discarded samples were normalized to 33%.

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Table 6
Results of the Bayes Factor comparisons using the stepping-stone estimation procedure for the Marginal lnL. Positive values indicate support for M_1 (rows) over M_0 (columns) models. Significant supports (strong evidence) are given bold.

Marginal lnL	Partition Strategy		M_0 MinPart	MidPart	FullPart
−34184.3135	M_1	MinPart	–		
−32278.7985		MidPart	**3811.029**	–	
−32173.0100		FullPart	**4022.607**	**211.577**	–

distribution (>200; Drummond and Rambaut, 2007), were significantly lower for the more complex partitioning strategies. This might well be correlated with the decrease in support values for these more complex strategies.

Discussion

Monophyly and internal relationships within the Mesembrinellidae

The monophyly of Mesembrinellidae, as recovered in the analyses, is well supported in the literature (Guimarães, 1977; Toma and Carvalho, 1995; Rognes, 1997; Bonatto, 2001). Morphological character states currently supporting the monophyly of the family include: (1) metathoracic spiracle with a single, large, reniform lappet, with dorsal opening; (2) anterior spiracle with drop-like shape with dorsal opening; (3) spermathecae elongated, each forming a long sclerotized tube; (4) epandrium and surstylus fused; (5) 8th sternite absent in females; and (6) female post-abdomen not forming a telescopic ovipositor. Other possible character states supporting the group—in combination with the ones previously listed—are the presence of interfrontal bristles in the females (Rognes, 1997) and the macro (uni) larviparous habit (Pape, 1992).

It should be clear that there is a weak taxon sampling of the species-rich outgroups, so the relationships obtained for the position of the Mesembrinellidae within the Oestroidea should be considered carefully. The considerably larger taxon sampling of the ingroup —22 of the 36 known species—suggests much more reliable results for the topology at this level. Nevertheless, based on the results found in our analyses, there seems to be enough evidence to corroborate the hypothesis that the Mesembrinellidae do not form a monophylum with the family Calliphoridae, which is undoubtedly para or polyphyletic (Rognes, 1997). The separation of the Mesembrinellidae from the monophyletic core calliphorid group (comprising the subfamilies Calliphorinae, Luciliinae, Melanomyinae, Toxotarsinae and Chrysomyinae) is even more obvious. Hence, the proposition of Guimarães (1977) of giving family status to the group seems well corroborated, with evidence from both morphological and molecular data (Rognes, 1997; Kutty et al., 2010; Marinho et al., 2012; Singh and Wells, 2013; Winkler et al., 2015), and is supported here.

For the relationships inside the family, there is agreement and some discrepancies with previous studies based on morphological information. The inferred sister-group relationship between *Laneella* and the remaining Mesembrinellidae agrees with the findings of Guimarães (1977), Toma and Carvalho (1995) and Bonatto (2001). The main character supporting this position is the shape of the spermathecae, considered plesiomorphic for the family by all these authors. In this context, the position of *M. patriciae* as sister of *Laneella* was, at first sight, unexpected. *Mesembrinella patriciae* was described by Wolff (2013) and, thus, was not in Toma and Carvalho's (1995) or Bonatto's (2001) taxon sampling. The inclusion of *M. patriciae* in *Mesembrinella* was based originally on the presence of the diagnostic combination of external character states for the genus: (1) humeral callus with three bristles; (2) presence of post-humeral bristles; and (3) presence of 2–3 katepisternal setae (Guimarães, 1977; Wolff, 2013). These characters states are, nevertheless, variable among the species of the genus and some of them can be found in other mesembrinellid genera as well (Bonatto, 2001). The position of *M. patriciae* obtained here suggests that it should be transferred to *Laneella*. We examined the female reproductive tract (not described in the original publication) and found a *Laneella*-type ("tuberiform") spermatheca (Fig. S6) that clearly supports the position recovered for the species in our analyses. The shape of the spermathecae, hence, as proposed by Guimarães (1977), is proven to be a reliable character (in the sense of not having wider homoplastic origins within the family) and its states identify more or less derived clades within the family (Fig. S1). At the same time, the confirmation that *M. patriciae* has a tuberiform spermatheca points to the predictive power of the inferred phylogeny. The presence of metallic reflections in the abdomen of *M. patriciae*, as well as of other external features referred to above, highlights the need for a revision of the diagnosis of the genus *Laneella*.

Regarding the relationships among the remaining lineages of the family, the position of *Eumesembrinella* nested inside a paraphyletic *Mesembrinella* is in disagreement with previous morphological studies. *Eumesembrinella*, in those studies, appears in a clade with *Huascaromusca* and *Giovanella* (in addition to *Thompsoniella* and *Henriquela*, not sampled here). The major morphological character states supporting a clade composed of these genera are the presence of a row of

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discal lateral setae on abdominal tergite 1 + 2 and the undeveloped facial carina—considered a reduction from the developed one found in the ground plan of the family (Toma and Carvalho, 1995; Bonatto, 2001). This latter feature was, however, interpreted differently by Rognes (1997), who considered the presence of a strongly developed facial carina only in the Ameniinae and Oestridae, coding the entire taxon Mesembrinellidae in his data matrix as undeveloped. Nevertheless, both states can be found in other species of Mesembrinellidae in different, though similar, configurations. For example, *M. bicolor*, recovered here as sister species of the genus *Eumesembrinella*, was described by Bonatto (2001) as having a slightly developed carina (which also occurs in *M. bellardiana*) and the presence of large setae in the lateral portion of tergite 1 + 2 (but not arranged in a row and not as developed as in *Eumesembrinella*). Apparently, these features are plastic and may have developed more than once in the evolution of the group. Additional analyses may suggest that *Eumesembrinella* should be merged with *Mesembrinella* as a junior synonym.

Inside *Eumesembrinella*, Toma and Carvalho (1995) found *E. cyaneicincta* as sister of a clade composed of (*E. quadrilineata* + (*E. randa* + *E. benoisti*)), whereas Bonatto (2001) recovered *E. quadrilineata* as the sister of the remaining species of the genus. Guimarães (1977) considered the existence of two distinct subspecies of *E. cyaneicincta*—*E. cyaneicincta cyaneicincta* (Surcouf, 1919) and *E. cyaneicincta pauciseta* (Aldrich, 1922)—respectively for the populations in south-east and north-west Brazil. According to Guimarães (1977), they lack any distinctive features in the male genitalia, but can be separated by the tibia colour pattern and the presence/absence of post-humeral setae. The relationships recovered here suggest that these are actually two separate species, with the SE group being sister to the entire NW clade of *Eumesembrinella*. Hence, *E. pauciseta* should be elevated to species status, whereas the name *E. cyaneicincta* should be applied only to the SE clade. A formal treatment for the nomenclatural changes mentioned in this paper will be published elsewhere.

Regarding the relationships within *Mesembrinella*, both Toma and Carvalho (1995) and Bonatto (2001) recovered most species of the genus in a polytomy. *Mesembrinella peregrina* was the only species recovered by Toma and Carvalho (1995) out of the polytomy, sister to all remaining Mesembrinellinae (*sensu* Guimarães, 1977). This position was supported by two character states: (1) tergites 6 and 7 + 8 not fused, differing from all remaining species in mesembrinellines; and (2) presence of small teeth-like projections at the apex of the dorsolateral process of the phallus (formerly, the aedeagus), a feature also found in *Eumesembrinella*. Revising the material used by Toma and Carvalho (1995), Bonatto (2001) found that the condition of tergites 6–8 in *M. peregrina* did not differ from that of other species in the genus (all fused) and that teeth-like projections can also be found at least in *M. pictipennis* (but not in *M. bicolor*). This shows that this character is quite plastic and we should be careful with previous inferences based on this feature. The position of *M. peregrina* in our analyses as sister to (*Eumesembrinella* + *Mesembrinella*) lacks morphological support.

Within *Mesembrinella*, Bonatto (2001) recovered a clade comprising *M. townsendi*, *M. batesi*, *M. appolinaris*, *M. pictipennis*, *M. currani*, *M. brunnipes* and *M. umbrosa* (the latter two not sampled in this study). This was suggested by the absence of a marginal row of setae in the abdominal tergite 4, a reversal to the plesiomorphic condition in the family (Bonatto, 2001). This clade was also recovered here in all analyses conducted, as sister to *M. bellardiana*, with moderate support (MP BS = 68/88; ML BS = 76/85/78; BI PP = 0.92/0.82/0.61). It is noteworthy that this absence of a marginal row of setae is also found in other species of Mesembrinellidae, such as some *Eumesembrinella* and *Giovanella*, again demonstrating a quite high level of homoplastic evolution of characters in the group.

Finally, for the species *M. bellardiana*, the two "populations" sampled here (SE and NW) were recovered as part of the same clade, but with considerable divergence between them. As is the case for *E. cyaneicincta*, Guimarães (1977) also proposed two subspecies in *M. bellardiana*: (1) *M. bellardiana bellardiana* (Aldrich, 1922) and (2) *M. bellardiana fuscicosta* (Seguy, 1925), distributed in southern and northern parts of South America, respectively. According to Guimarães (1977), they could be distinguished by the femur coloration and head pollinosity, despite lacking any conspicuous differences in the male genitalia morphology. Bonatto (2001), in his revision of the group, expanded the known distribution for the species to include Venezuela and the Brazilian states of Paraná, Pará and Rondônia, in some cases with both subspecies coexisting in the same locality. Our analyses, with specimens sampled only from the two extremes of the distribution, suggest that these two subspecies might well comprise two distinct species, a fact further supported by the analyses of genetic divergence data of both *COI* and *ITS2* regions.

A better understanding of the relationships inside the *Huascaromusca* + *Giovanella* clade is still affected by taxonomic sampling limitations, namely the lack of sequences for some *Huascaromusca* species and for the small genera *Henriquela* and *Thompsoniella*. It is worth mentioning that species of these genera are relatively scarce in collections and very hardly collected in the field. The monophyly of a clade (*Huascaro-*

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musca + *Giovanella*) with high support in our ML and BI analyses suggests that the character state usually considered to group these species—the presence of a row of discal setae on abdominal T5—may have a single origin in the evolution of the family. The inclusion of *G. carvalhoi* inside *Huascaromusca* should actually not be a surprise, because it also shares this feature (Wolff et al., 2013). There seems to be sufficient grounds to accept *Huascaromusca* and *Giovanella* as synonyms, something that might be applicable also for *Henriquela* and *Thompsoniella*. For relationships among the species of *Huascaromusca*, a close relationship between *H. vogelsangi*, *H. aeneiventris* and *H. purpurata* was already proposed by Bonatto (2001) based on the presence of violet bands on the posterior portion of abdominal tergites. This clade was also recovered in the ML and BI analyses in this study.

Effects of data partitioning on phylogenetic analyses

Data partitioning based on gene regions is now a common practice in phylogenetic analyses and its use is justified by the better modelling of the distinct time and mode of evolution of the different genes, even those that are more or less linked (Blair and Murphy, 2011; Lanfear et al., 2012). Further refinements of partition schemes, dividing gene regions in its constituent heterogeneous parts (i.e. codon positions and structural or functional motives) are biologically reasonable, but there is an intrinsic problem of increased parametric space that needs to be accounted for, because it demands more computational time and resources. Also, parameter-rich models and partition schemes can be associated with the risk of an increased occurrence of stochastic errors due to the smaller number of sites retained in each partition and the multiplication of errors during the estimation process of multiple parameters (Blair and Murphy, 2011).

The biological reasonability of further dividing gene partitions has statistical support, usually leading to higher marginal lnL values and, thus, to a better fit of the model to the data in analyses with more complex partitioning strategies (Nylander et al., 2004; Brandley et al., 2005; Petkovits et al., 2011; Marinho et al., 2012). The increment in parametric space when using highly partitioned models, nevertheless, is accompanied by increased uncertainty in topology estimation and, consequently, in decreased overall support for the inferred relationships, as was found in a previous work in Oestroidea based on a similar dataset (Marinho et al., 2012). In this sense, an intermediate partition scheme, such as the MidPart strategy used here (as defined by the PartitionFinder software, which combined smaller *ad hoc* defined partitions across gene regions), may be preferred, because it still accounts for heterogeneity in evolution inside gene regions while avoiding overparameterization (Lanfear et al., 2012). This is supported by the increase, albeit small, in the average PP support values in the MidPart scheme when compared with the MinPart and FullPart schemes.

Although the use of more complex partitioning strategies on average seems advantageous, the use of different partitioning schemes is usually associated with few, if any, topological changes among inferred trees, which are usually restricted to weakly supported relationships (Brandley et al., 2005; Marinho et al., 2012). In our analyses, these topological changes were restricted almost exclusively to the outgroup taxa and largely associated with the erratic positioning of *O. ovis*. Among the ingroup terminal taxa, *M. peregrina* is the only exception, but its position in the tree is also variable among reconstruction methods (especially between the ML/BI and the MP). The real effects on topology estimation of using different partition schemes are yet to be more fully understood, but the scenario so far depicted indicates that it may at least reveal problematic taxa in the analysis and point to parts of the tree that still need a better taxon and/or molecular markers sampling.

In a consideration of the use of RNA secondary structure substitution models for phylogenetic reconstruction, Letsch et al. (2010) found that although considering structural information on the alignment procedure for RNA regions is undoubtedly advantageous, the use of mixed DNA/RNA models for phylogenetic reconstruction showed different results in different analyses and further studies are still necessary. Because the two more complex partition schemes used here, the MidPart and FullPart strategies, included RNA secondary structure models, their discussion and following conclusion may partly overlap here.

Finally, it is worth mentioning that although more complex partition schemes are associated with larger parametric spaces and thus require more computational time and power for analyses, our results show that the MCMC runs under the most complex model (FullPart) took considerably less time (in number of generations) to converge than the runs under other partition schemes. This, together with the fact that both duplicates recovered the same number of trees in the 95%/99% confidence interval and the same topology for the 50% majority rule consensus tree (i.e. probably reached the same optimum), suggests a "cleaner" parametric space without too many competing local optimums (assuming both runs have reached a global optimum). Further evidence pointing to the same direction comes from the fact that both runs under less complex partition schemes, that took more than 10 million generations to converge (MinPart A and MidPart B), actually "converged" much earlier to

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a local optimum, but then a swap in the chains led to a more denser, perhaps "global" optimum (data not included here). As pointed out in previous studies, the effects and real benefits of the use of complex partition schemes for phylogenetic inference are still controversial and further studies are needed.

Perspectives

The reliable, robust phylogeny for the family obtained with this study now allows some additional investigations and approaches to be performed. From a taxonomic point of view, collecting and sequencing *Henriquela*, *Thompsoniella*, *Albuquerquea* and *Souzalopsiella* are priorities to solve nomenclatural and phylogenetic issues in the system for the family. This approach is also necessary for some of the widespread nominal species apparently including more than one biological species: proper geographical sampling and sequencing would help to estimate the degree of divergence between these populations, leading to a better understanding of the speciation patterns of calyptrate flies in the tropics. Finally, the occurrence of viviparity in the Mesembrinellidae family makes it an interesting biological model for very important studies on the evolution of reproduction and development.

Conclusions

The monophyly of Mesembrinellidae has never been questioned and is assured here based on the first molecular study. Additionally, the taxon sampling used here is enough to corroborate that the clade corresponds to a lineage cladistically removed from the core calliphorids. A robust hypothesis for the position of the family, however, depends on a much more detailed sampling of some of the speciose Oestroidea families.

Phylogenetic relationships among the mesembrinellids proposed so far based on morphological characters showed lack of resolution and low support for recovered clades. The relationships obtained in this study, based on a fairly good number of sequences and taxa, have good resolution to parts that were poorly supported in previous analyses. Based on our findings, *Eumesembrinella* should be synonymized with *Mesembrinella* and *Giovanella* should be synonymized with *Huascaromusca*. *Laneella* is a taxon of generic rank that is informative in the family and its diagnosis must be emended to also include *M. patriciae*. This emendment will require a more refined study of the external morphology of the genus. In a separate taxonomic paper we will formally propose the nomenclatural acts demanded by this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Matrix S1. Data matrix (concatenated alignment) used in the phylogenetic inference analyses.

Fig. S1. Proposed phylogenetic relationships among mesembrinellids based on morphological characters.

Fig. S2. (a) Secondary structure model for domains IV and V (3′ end) of the 16S rDNA of *Laneella nigripes*. Dots represent nucleotides in regions not sequenced in this study, shown for visual purposes. Structural model is based on the one described for *D. melanogaster* by Cannone et al. (2002). Numbers on helix regions follow the nomenclature proposed by Gillespie et al. (2006). (b) Structural variation on helix H2077 for some of the species sampled in this study. (c) Predicted secondary structure for the region including helix H2347 - bases 371 to 394 in (a) – for some of the species sampled in this study.

Fig. S3. (a) Secondary structure model for domains I and II (5′ end), including expansion domains D1, D2 and D3, of the 28S rDNA of *Laneella nigripes*. Dots represent nucleotides in regions not sequenced in this study, shown for visual purposes. Structural model is based on the one described for *D. melanogaster* by Cannone et al. (2002). Numbers on helix regions follow the nomenclature proposed by Gillespie et al. (2006) (b), (c) Structural model for expansion domains D2 and D3, respectively, of some of the species sampled in this study.

Fig. S4. Predicted ITS2 secondary structures for some Mesembrinellidae species. Nomenclature for helix-domain regions are based on the one proposed for *D. melanogaster* by Young and Coleman (2004) and used in Marinho et al. (2012).

Fig. S5. Phylogenetic relationships inside the Mesembrinellidae clade as inferred in the (a) BI-FullPart, (b) ML-FullPart and (c) MP analyses.

Fig. S6. Female reproductive tract of *Laneella nigripes* (a), *Mesembrinella peregrina* (b), and *Mesembrinella patriciae* before (c) and after (d) treatment with a 10% KOH solution to remove fat tissues from the spermathecae (spmth).

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