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## Molecular phylogeny of Blaberidae (Dictyoptera, Blattodea), with implications for taxonomy and evolutionary scenarios

Frédéric LEGENDRE<sup>1,\*</sup>, Philippe GRANDCOLAS<sup>2</sup> & France THOUZÉ<sup>3</sup>

Institut de Systématique, Évolution, Biodiversité ISYEB – UMR 7205 – MNHN  
CNRS UPMC EPHE, Muséum national d’Histoire naturelle,  
Sorbonne Universités, 57 rue Cuvier, CP 50, 75005 Paris, France.

\* Corresponding author: [legendre@mnhn.fr](mailto:legendre@mnhn.fr)

<sup>2</sup> Email: [pg@mnhn.fr](mailto:pg@mnhn.fr)

<sup>3</sup> Email: [francethouze@gmail.com](mailto:francethouze@gmail.com)

<sup>1</sup> [urn:lsid:zoobank.org:author:8B1163C9-338E-425A-9107-C185D4AB415F](https://zoobank.org/author:8B1163C9-338E-425A-9107-C185D4AB415F)

<sup>2</sup> [urn:lsid:zoobank.org:author:FCCF6C9C-F929-453E-989B-C5BB4B04B54A](https://zoobank.org/author:FCCF6C9C-F929-453E-989B-C5BB4B04B54A)

<sup>3</sup> [urn:lsid:zoobank.org:author:6692EB45-5E95-41AB-BB1F-59B3154B3402](https://zoobank.org/author:6692EB45-5E95-41AB-BB1F-59B3154B3402)

**Abstract.** In the present “tree-thinking” period, relying on accurate phylogenetic hypotheses is of paramount importance for biologists interested in an evolutionary perspective. In the Blaberidae cockroaches, a well-defined monophyletic family comprising several model species, no such phylogenetic tree is available despite several earlier contributions. Here, using six molecular markers (12S, 16S, 18S, 28S, COI and COII), we investigate the relationships of Blaberidae and compare our results with the traditional morphology-based classification. This resulted in a broad spectrum of situations, from congruent and well-supported hypotheses (e.g., the monophyly of Blaberidae, Oxyhaloinae and (Geoscapheinae + Panesthiinae)) to incongruent and weakly supported results (e.g., polyphyly of Perisphaerinae). We emphasize that interesting and contrasted situations lie between the two extremities of this spectrum, especially concerning the genera *Thanatophyllum* Grandcolas, 1991, *Phoetalia* Stål, 1874, *Laxta* Walker, 1868 and *Pronauphoeta* Shelford, 1909. We also discuss the phylogenetic position of two *incertae sedis* genera (*Eustegasta* Gerstaecker, 1883 and *Gynopeltis* Gerstaecker, 1869). We conclude that in-depth signal analyses should be performed to better understand molecular evolution and its consequence on tree reconstruction for this group. As for phylogenetic relationships *per se*, new markers should be searched for, especially to decipher deeper relationships in Blaberidae.

**Keywords.** Biogeography, incongruence, genitalia, morphology, phylogenetic signal.

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### Introduction

We are presently experiencing what has been called a “tree-thinking biology” era (Grandcolas *et al.* 1994; O’Hara 1997; Baum *et al.* 2005; Gregory 2008; Omland *et al.* 2008; Baum & Smith 2013; Losos

*et al.* 2013). Myriad phylogenies are produced every year (Parr *et al.* 2012) and these hypotheses serve to investigate diverse evolutionary questions or to revise classifications. Even though these numerous hypotheses might be contradictory and thus bewildering (Chiapella *et al.* 2014), they are essential for present and future studies, especially when they involve model organisms from various disciplines.

Blaberid cockroaches comprise model species in research about biomimetics (e.g., Nelson *et al.* 1997), physiology (Li 2007), sociality (Costa 2006; Pellens *et al.* 2007) or reproductive behavior (Roth & Willis 1954; Banerjee *et al.* 2016). These fields of research would benefit from a clear phylogenetic hypothesis or a phylogeny-derived robust classification. Yet, such a phylogeny is still lacking despite several relevant contributions since Saussure (1864) erected the Blaberidae family.

McKittrick (1964) was the first to define this family as we consider it today. She distinguished three complexes which she named blaberoid, epilamproid and panchloroid, but without performing any formal phylogenetic analysis. A cladistic analysis of her data, however, revealed that her blaberoid and panchloroid complexes were not monophyletic (Legendre 2007). Later, cockroach systematists identified several morphological synapomorphies supporting the monophyly of Blaberidae (Grandcolas 1996; Klass & Meier 2006; brood sac underneath the female genitalia, sclerite R3v small, first vein of vannus with many basal rami, tracheation very dense and tracheae vesicle-shaped, presence of a muscle connecting sclerite L2 and pouch lve). Monophyly has also repeatedly been found with molecular data as well (Maekawa *et al.* 2003; Inward 2007; Pellens *et al.* 2007; Djernaes *et al.* 2012; Legendre *et al.* 2014, 2015).

Within Blaberidae, however, the phylogenetic relationships remain controversial, despite early morphological studies (e.g., Grandcolas 1993, 1997). The only largely supported results are the close relationships between Zetoborinae and Blaberinae on the one hand, and Panesthiinae and Geoscapheinae on the other. The position of Panchlorinae as the first diverging lineage within the family also seems to be consistently found lately even though often based on a limited taxon sample (Legendre *et al.* 2014, 2015). The phylogenetic positions of the seven remaining subfamilies still need to be investigated further, as do the phylogenetic positions of 24 *incertae sedis* genera (according to the Blattodea Species File, BSF; Beccaloni 2014).

The taxonomy of cockroaches relies heavily on morphological characters, especially those of the genitalia. Insect taxonomists often use genitalia for species identification as well as for classification, proposing synapomorphies for diverse subfamilies and genera (e.g., Grandcolas 1993, 1994, 1996, 1997; Roth 2003; Klass & Meier 2006). The current classification systems of cockroaches are thus based mainly on morphological characters (Roth 2003). However, like any set of characters, morphological characters benefit from completion with other kinds of data, as already shown when combining molecular and behavioral characters for instance (Legendre *et al.* 2014).

In terms of geographical range, Blaberidae have a pantropical distribution. Some subfamilies are restricted to one continent, while others encompass several. Blaberinae and Zetoborinae are endemic to America (mainly South America), Gyninae and Oxyhaloinae are endemic to Africa, Geoscapheinae is endemic to Australia, and Paranauphoetinae is endemic to Asia. Panchlorinae is found in America and Africa, Pycnoscelinae is found in Asia and South America, while Diplopterinae and Panesthiinae are found in Asia and Australasia. Perisphaerinae is found in Africa, Asia and Australasia, and Epilamprinae is found in America, Africa, Asia and Australasia (Princis 1964; Beccaloni 2014).

In this paper, we investigate the phylogenetic relationships in blaberid cockroaches using six molecular markers. We then compare our results to the largely pre- or semi-phylogenetic current morphology-based classification of this family as presented in the CSF (Beccaloni 2014). We underline the differences found and interpret them in terms of potential morphological evolution and biogeography. We also propose future directions to improve our knowledge of the phylogenetics of Blaberidae.

## Material and methods

### Taxonomic and character sampling

We sampled 136 taxa, 128 of them belonging to 11 out of the 12 currently distinguished subfamilies of Blaberidae (according to the BSF; Beccaloni 2014): 18 Blaberinae (12 genera out of 23 genera recognized in the BSF, i.e. ~52%), one Diplopterinae (one genus, 100%), 26 Epilamprinae (at least 11 genera, ~24%), 10 Geoscaphinae (four genera, 100%), five Gyninae (one genus, 20%), 13 Oxyhaloinae (11 genera, ~65%), three Panchlorinae (one genus, 20%), 23 Panesthiinae (five genera, ~71%), 18 Perisphaerinae (at least seven genera, ~37%), two Pycnoscelinae (one genus, ~33%), and nine Zetoborinae (eight genera, 57%). We also included five specimens belonging to the *incertae sedis* genera *Eustegasta* Gerstaecker, 1883 and *Gynopeltis* Gerstaecker, 1869. Outgroup taxa comprise one Ectobiidae (*Paratropes* sp. 1), one Corydiidae (*Therea petiveriana* Linnaeus, 1758) and one Blattidae (*Blatta orientalis* Linnaeus, 1758) as the rooting outgroup taxon. Among Ectobiidae, all subfamilies have been postulated as being the sister group of Blaberidae, including Pseudophyllodromiinae (Grandcolas 1996; Inward *et al.* 2007; Pellens *et al.* 2007; Djernaes *et al.* 2012; Legendre *et al.* 2015). At first, we included several species of Pseudophyllodromiinae (*Anisopygia* sp., *Asemoblattana* sp., *Euphyllodromia* sp., *Latiblattella* sp. 3) but we then discarded them because of their artifactual position within Blaberidae (among Epilamprinae, data not shown). All the species of Pseudophyllodromiinae sequenced so far show highly modified sequences, which results in high percentages of divergence with ingroup species and may cause artifacts in tree reconstruction such as long branch attraction (Wheeler 1990; Bergsten 2005; Legendre *et al.* 2015). Also a couple of additional species of Blattellinae were used as outgroup taxa in preliminary analyses (*Ischnoptera* sp. and *Xestoblatta* sp.), but were not kept because of their missing data, which might alter tree reconstruction (e.g., Lemmon *et al.* 2013; Roure *et al.* 2013). Whatever the outgroup combinations used in our preliminary analyses, the supported results presented here were always retrieved.

We used six molecular markers to reconstruct blaberid phylogenetic relationships: 12S rRNA (~440 bp), 16S rRNA (~440 bp), 18S rRNA (~1900 bp), 28S rRNA (~2350 bp), cytochrome oxidase subunit I (COI, 1179 bp) and cytochrome oxidase subunit II (COII, 671 bp). Primers and molecular techniques were described in Legendre *et al.* (2008). We provided 175 new sequences, which were deposited in GenBank with the following accession numbers: KY497575–KY497749. In average, taxa were documented for ca 4000 bp (for a total alignment of 6991 bp). We sampled 92% of the taxa for 12S, 57% for 16S, 95% for 18S, 76% for 28S, 38% for COI and 78% for COII. The [Supplementary file](#) shows the details of the completion of our dataset.

### Phylogenetic analyses

All sequences were blasted prior to any analysis to check for potential contamination. Then, we computed alignments using MUSCLE (Edgar 2004) as implemented in Seaview v. 4 (Gouy *et al.* 2010). For 28S, alignments were performed separately for each fragment and then concatenated, whereas fragments were concatenated before alignment for 18S. The 28S fragments do not or hardly overlap so that they could not be aligned altogether. For each marker, the proposed alignments were sometimes dubious, especially in the terminal regions because of fragments that were not always congruent in coverage. We corrected these problems by refining the alignment manually. Moreover, we ensured that alignments respect the codon protein reading frame for COI and COII. Once we obtained separate alignments for the six markers, we used SequenceMatrix v. 1.7.8 (Vaidya *et al.* 2011) to produce the full dataset composed of 6991 bp.

We performed separate and combined analyses in a probabilistic framework. For separate analyses, we selected the best model that does not mix I (proportion of invariant sites) and G (heterogeneity of substitution rates) using jMrModeltest v. 2.1.3 (Darriba *et al.* 2012) with the AICc criterion.

For combined analyses, we used Partitionfinder v. 1.1.1 (Lanfear *et al.* 2012; options: *branchlengths=linked*, *model\_selection=AICc* and *search=greedy*) to select the best partition strategy among models supported by MrBayes. The best partitioning strategy involved nine partitions: one partition with a GTR+G model for each non-protein-coding marker; four distinct partitions for the first two positions of COI and COII with a GTR+G model; and a common partition for the third positions of COI and COII, also with a GTR+G model.

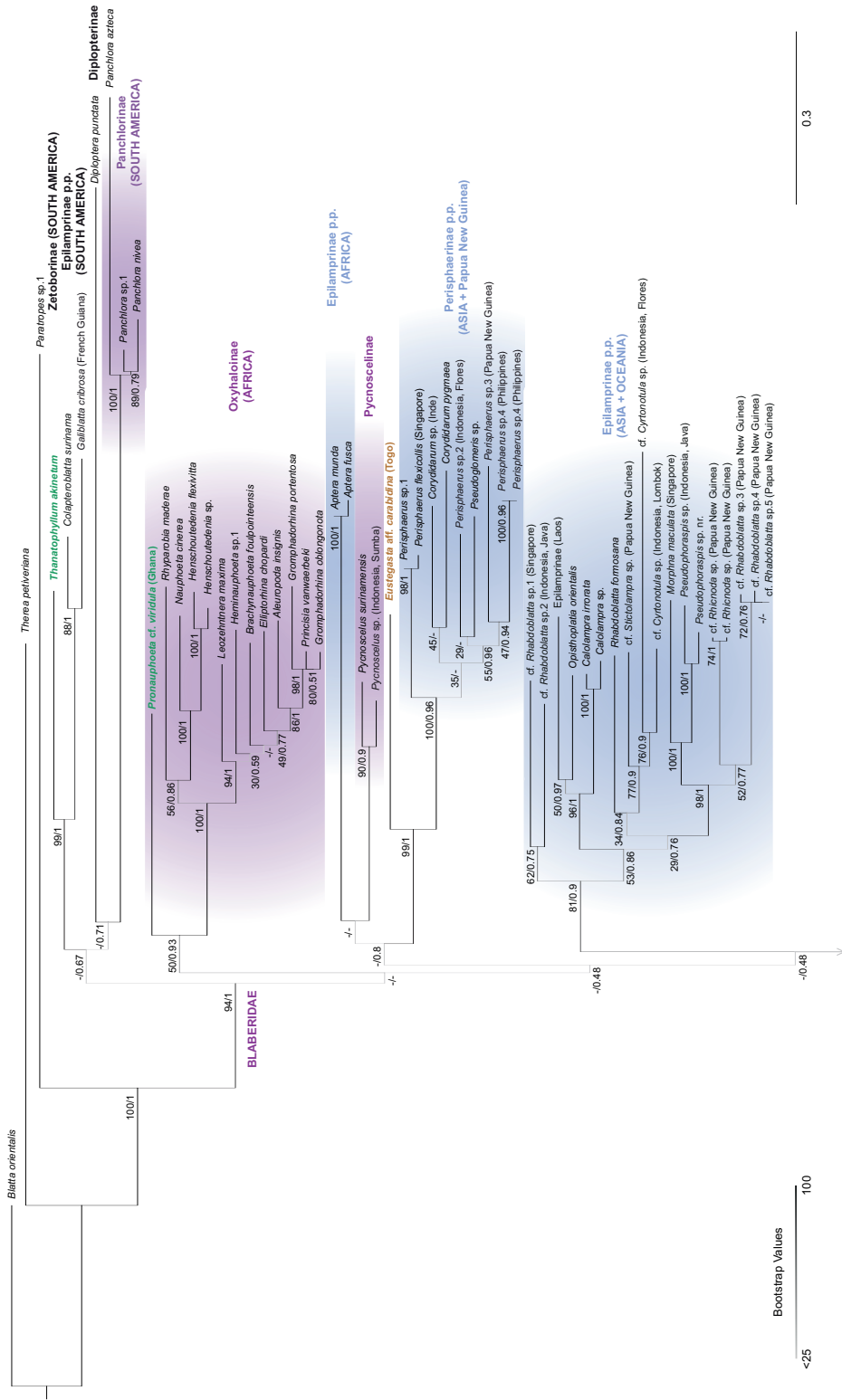
In Maximum Likelihood (ML), we used RAxML v. 8.2.4 (Stamatakis 2014) to reconstruct phylogenetic hypotheses, whereas we used MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) in Bayesian Inferences (BI). ML analyses were performed using the rapid hill-climbing algorithm for 500 replicates. We then computed 500 bootstrap replicates. In BI, we launched two runs of four chains each until the average deviation of split frequencies reached a value inferior to 0.01 (options: *stoprule=yes*, *stopval=0.01*). We then assessed convergence using Tracer v. 1.6 (Rambaut *et al.* 2014), checking that ESS values all exceed 200.

## Results

The best tree from the ML combined analysis is shown in Fig. 1 and is very similar to the tree reconstructed in Bayesian Inference (note that convergence diagnostics and ESS values were satisfying, except for the ESS values of the rate multiplier parameters). Blaberidae were monophyletic with a moderate bootstrap support and a maximal posterior probability (BS = 94, PP = 1). Panchlorinae, Gyninae, Pycnoscelinae and Oxyhaloinae were monophyletic (BS/PP = 100/1, 99/1, 90/0.9 and 50/0.93, respectively), while Blaberinae, Epilamprinae, Perisphaerinae and Zetoborinae were polyphyletic. The group (Panesthiinae+Geoscapheinae) was monophyletic (BS = 99, PP = 1), with Australian *Panesthia* nested within Geoscapheinae.

Overall, this tree showed very weak support values for deeper relationships within Blaberidae. Nevertheless, some clades with geographic congruence were retrieved. African Perisphaerinae, Asian Perisphaerinae, African Epilamprinae and Asian-Oceanian Epilamprinae (except *Rhabdoblatta* cf. *circumdata* from Singapore) were monophyletic with high to moderate support values (BS/PP = 100/1, 100/0.96, 100/1 and 81/0.9, respectively), and a Neotropical clade composed of the Blaberinae and most of the Zetoborinae was found (BS/PP = 43/0.86; see exceptions below).

The phylogenetic position of *Phoetalia pallida* (Brunner von Wattenwyl, 1865) (Blaberinae) as sister species to *Schultesia lampyridiformis* Roth, 1973 (Zetoborinae) was highly supported (BS/PP = 100/1) and found in almost all the analyses performed (Fig. 2). Three other Blaberinae species (*Lucihormetica subcincta* (Walker, 1868), *Hormetica* sp. and *Blaptica* sp.) were found closer to most species of Zetoborinae than to other Blaberinae. *Parasphaeria boleiriana* Grandcolas & Pellens, 2002 (Zetoborinae) branched outside of the Neotropical clade composed of the Blaberinae and most of the Zetoborinae, but its phylogenetic position was unsupported (BS/PP = 11/0.81), whereas the position of *Thanatophyllum akinetum* Grandcolas, 1991 (Zetoborinae) as sister group to some Neotropical Epilamprinae was highly supported (BS/PP = 99/1) and found in half of the separate analyses (Fig. 2). Within Oxyhaloinae, the Nauphoetini were monophyletic, whereas the Gromphadorhini were monophyletic only if the *incertae sedis* genera *Brachynauphoeta* van Herrewege, 1975 and *Heminauphoeta* Saussure, 1891 are taken into account. The *incertae sedis* Oxyhaloinae genus *Pronauphoeta* was sister group to all other species of Oxyhaloinae sampled here (BS/PP = 50/0.93), but with other positions in three one marker analyses (12S, 16S and 18S; Fig. 2). Concerning Perisphaerinae, *Laxta* sp., the only Australian individual sampled, was sister group to African Perisphaerinae (BS/PP = 32/0.93), a result almost only found with the combined dataset (Fig. 2).



**Fig. 1.** [part 2 on next page] Optimal phylogenetic tree reconstructed in Maximum Likelihood with the combined dataset. Bootstrap values and posterior probabilities are reported for each node (bootstrap values below 25% are not displayed). The color of internal branches is proportional to bootstrap values. Geographic origin of the specimens sequenced is provided in brackets after the species names. In purple, monophyletic group congruent with morphological hypotheses; in blue, monophyletic group with geographic consistency at the continental level; in brown, *incertae sedis* species; in green, four species with conflicting and supported positions (*Thanatophyllum akinetum* Grandcolas, 1991 and *Phoetalia pallida* (Brunner von Wattenwyl, 1865) or congruent, but weakly supported positions (*Laxta* sp. and *Pronauphoeta* cf. *viridula* (Palisot de Beauvois, 1805)). The four latter species are discussed in the text (see also Fig. 2). The subfamilies indicated on the right of the tree are derived from traditional morphology-based classifications. Units for the branch length scale at the bottom right: number of expected substitutions per site.

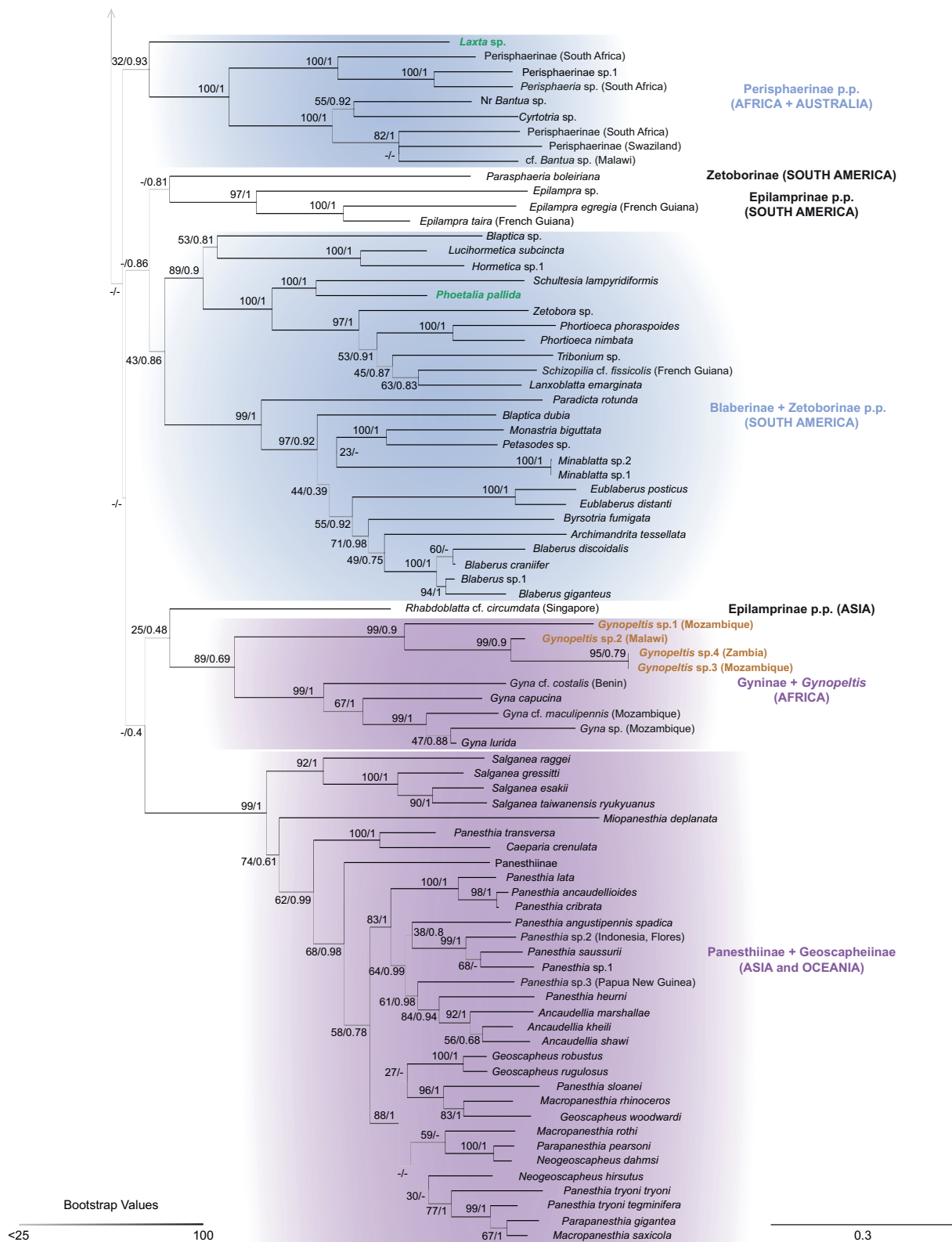


Fig. 1. Part 2. See legend on preceding page.

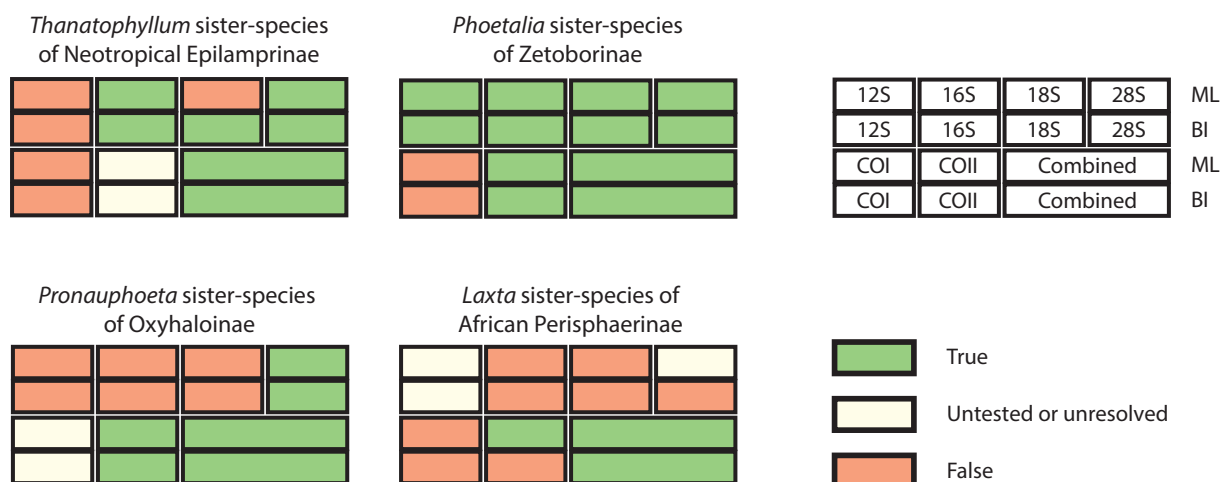
Two *incertae sedis* genera were included in the analyses: *Eustegasta* aff. *carabidina* had Asian Perisphaerinae as its sister group (BS/PP = 99/1); *Gynopeltis* spp. were monophyletic (BS/PP = 99/0.9) and sister group to Gyninae (BS/PP = 89/0.69).

## Discussion

Like for many different taxonomic groups, the traditional classification of Blaberidae relies mainly on morphological characters. Twelve subfamilies have been delimited so far, while 24 genera remain of unknown affinity. We sampled 11 of these subfamilies with molecular data to investigate the phylogenetic relationships of the family, which should be useful for classification and evolutionary studies in this cockroach family. We ended up with a broad-spectrum situation regarding congruence of our molecular phylogenetic hypothesis with the traditional morphology-based classification.

On the one side lie molecular hypotheses highly supported and congruent with morphology-based hypotheses. These unsurprising results have been suggested several times and concern the monophyly of Blaberidae, (Geoscapheinae + Panesthiinae) and Oxyhaloinae (Maekawa *et al.* 2003; Inward 2007; Pellens *et al.* 2007; Djernaes *et al.* 2012; Legendre *et al.* 2014, 2015). Likewise, a strong support was found, here and in previous studies, for Gyninae, Panchlorinae and Pycnoscelinae. It is worth noting though that for these subfamilies, only one genus was sampled, which is insufficient to test the monophyly of these subfamilies.

On the other side of the spectrum lie conflicting but poorly supported hypotheses. We do not discuss these results in depth because any conclusion about these groups would be premature. Indeed, given that deeper nodes were not supported, it is impossible to rule on the monophyletic status of the subfamily Perisphaerinae, for instance. Admittedly, African and Asian Perisphaerinae form two distinct and highly supported monophyletic groups. They were never found as sister groups, but none of their potential sister-group relationships were supported (BS < 35, PP < 0.73), so assessing that they cannot be sister groups would be ill-advised. Similarly, Epilamprinae is split into four clades (and one isolated species) with some geographic consistency and with moderate to high support, but its sister-group relationships are weakly supported (BS < 25, PP < 0.73). It is thus impossible to reach conclusions about these subfamilies with the molecular data at hand.



**Fig. 2.** Conflicting results among different molecular markers. The phylogenetic relationships of four species are detailed for separate and combined analyses performed in Maximum Likelihood (ML) and Bayesian Inference (BI). Boxes colored as ‘Untested or unresolved’ refer to missing data and multifurcation, respectively.



Interestingly, none of the molecular markers used here were informative enough to decipher the relationships among the major well supported blaberid clades, which remain poorly supported. This pattern does not seem to result from conflicting signals among molecular markers, because none of the separate analyses show robust deep relationships. Instead, it suggests that the molecular markers used are inefficient for deciphering these phylogenetic relationships. They appear uninformative for this period, which could suggest that a radiation occurred early on in the evolution of Blaberidae (probably during the Jurassic; Legendre *et al.* 2015), even though this remains to be demonstrated. Searching for new markers, as well as performing signal analyses, should help in making progress on this complicated issue (Whitfield & Lockhart 2007). Conversely, it is worth noting that most of the clades found have a strong geographical coherence. We already mentioned Epilamprinae and Perisphaerinae but, interestingly, Geoscaphinae, which are Australian, are not monophyletic because Australian *Panesthia* are nested among them. With their pantropical distribution and their ancestry tracking back to the Mesozoic, Blaberidae can be a relevant model to investigate complex biogeographic scenarios. Nevertheless, the present analysis shows that, before elaborating on them, more robust knowledge of relationships of subfamilies is needed, an issue already raised before (Legendre *et al.* 2014).

Between the two extremities of this spectrum are arguably the most interesting situations: conflicting but highly supported results on one hand and congruent but poorly supported results on the other.

*Phoetalia* Stål, 1874 and *Thanatophyllum* Grandcolas, 1991 are two genera showing highly supported, but conflicting positions with morphology-based classifications, where they belong to the subfamilies Blaberinae and Zetoborinae, respectively. Here, on the basis of 5654 bp belonging to five markers (COII is missing), *Thanatophyllum akinetum* was found to be the sister species of some Neotropical species of Epilamprinae. This relationship was found in half of the separate analyses performed (for the other analyses, *T. akinetum* was found as the sister species of Asian Epilamprinae, Perisphaerinae or Zetoborinae). At the moment of description, *T. akinetum* was placed within Zetoborinae (Grandcolas 1991), a position later supported in a cladistic study using 74 morphological characters and sampling 10 genera of Zetoborinae, 18 of Blaberinae, five of Gyninae and two of Diplopterinae (Grandcolas 1993). As for *Phoetalia pallida*, it was found nested within Blaberinae in Grandcolas (1993). Here it was found as the sister species of a species of Zetoborinae (*Schultesia lampyridiformis*), a result also found in five out of the six separate analyses. This disagreement between two studies with different taxon and character samplings does not allow us to decide which hypothesis, and thus which evolutionary scenario, should be favored. Blaberinae are notably characterized by male genitalia with spines on the prepuce (Roth 1970c). This character could be a synapomorphy of Blaberinae (including *P. pallida*), a striking convergence between *P. pallida* and Blaberinae (without *P. pallida*), or, depending on the phylogenetic structure of deeper nodes that remain to be deciphered, these spines could actually be a symplesiomorphy of a Neotropical clade comprising Blaberinae and most of the species of Zetoborinae. Solving these issues for these two species will require morpho-anatomic work at the Blaberidae scale, building on previous studies (e.g., Grandcolas 1991, 1993; Roth 1970a, 1970b, 1970c), and/or in-depth signal analyses of molecular data (Thouzé *et al.* in prep.).

Two results are congruent with morphology-based classification but with low support in molecular analyses. *Pronauphoeta* sp. (Oxyhaloinae) and *Laxta* sp. (Australian Perisphaerinae) were found to be the sister species of all other Oxyhaloinae and African Perisphaerinae, respectively. *Pronauphoeta* Shelford, 1909 was first placed by Shelford (1909) as intermediate between *Panchlora* Burmeister, 1838 (Panchlorinae) and *Nauphoeta* Burmeister, 1838 (Oxyhaloinae) but was later suggested to be closer to Oxyhaloinae (Rehn 1932; Kumar 1975) than to Panchlorinae, a hypothesis supported here in the combined analyses and with the markers 28S and COII (with the other markers, *Pronauphoeta* sp. was found as the sister species of some Epilamprinae, Perisphaerinae or in a much deeper position). At the tribe level, *Pronauphoeta* is of unknown affinity and, if its phylogenetic position were to be confirmed,

would belong to a new tribe. As for *Laxta* Walker, 1868, Grandcolas (1997) revised the Perisphaerinae and inferred that it belonged to this subfamily given one external morphological character and four genitalic characters. Molecular data suggest that *Laxta* is closely related to African Perisphaerinae. Nevertheless, its phylogenetic position fluctuates in the different separate analyses, with, for instance, diverse deep positions within the tree based on 18S (ML and BI), 28S (BI), COI (BI) or COII (BI). This result is a remarkable example of disagreement between separate and combined analyses, where signal from the separate analyses disappears in the concatenated analysis (Sousa *et al.* 2013; Thiergart *et al.* 2014), a phenomenon that remains to be understood. Sampling other Australian species, notably from the genus *Neolaxta* Mackerras, 1968, will be necessary to confirm the phylogenetic position of *Laxta* and later suggest biogeographic scenarios for this subfamily, presently known from three continents (Asia, Africa and Oceania; Grandcolas 1997).

We provide here the first taxonomic affiliation based on molecular data for the *incertae sedis* genera *Eustegasta* and *Gynopeltis*. *Gynopeltis* was found, with strong support, as closely related to Gyninae. Other genera of Gyninae than *Gyna* must be sampled, however, to assess whether *Gynopeltis* belongs to the Gyninae or if another subfamily (Gynopeltinae) should be erected. *Eustegasta* has been suggested as belonging to the Perisphaerinae before it was removed from this subfamily (Roth 1973; Grandcolas 1997). Here this African genus, only documented for half of the marker, is found with strong support as a sister group to Asian Perisphaerinae.

By deciphering phylogenetic relationships in Blaberidae with six molecular markers, we ended up with phylogenetic hypotheses disagreeing, in part, with morphology-based classification. While some situations should be easily resolved with an increase in taxonomic sampling or a decrease in missing data (e.g., congruent results, but with weak support), others would require analyzing the phylogenetic signal of each molecular marker more in depth (e.g., the phylogenetic position of *Laxta*). Saturation plots and analyses of bias in nucleotide composition should be performed with phylogenetic informativeness and network analyses (Huson & Bryant 2006; Townsend 2007; Philippe & Roure 2011). Also, given the long branches in the putative sister group Pseudophyllodromiinae (Legendre *et al.* 2015) and the results of our preliminary analyses and of previous studies on the effect of outgroup choice on tree reconstruction in Dictyoptera (Ware *et al.* 2008; Djernaes *et al.* 2012), the impact of outgroup choice in the phylogenetics of Blaberidae should be investigated further. Analyzing these conflicting signals and their causes remains, however, a complicated task, so cockroach phylogenetics would above all else benefit from new appropriate markers that are now reachable with the development of next generation sequencing methods (Metzker 2010). Markers informative for the Jurassic period should probably be targeted first to investigate blaberid phylogenetics.

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**Legend for [Supplementary file](#).** List of specimens used in this study, with information about the completion of our dataset. A color code is used to show the traditional taxonomic affiliation of the species sampled.