NATÁLLIA MARIA DE FREITAS VICENTE

BIOGEOGRAPHY, SYSTEMATICS AND EVOLUTION OF ACOUSTIC COMMUNICATION IN ENEOPTERINAE CRICKETS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae.*

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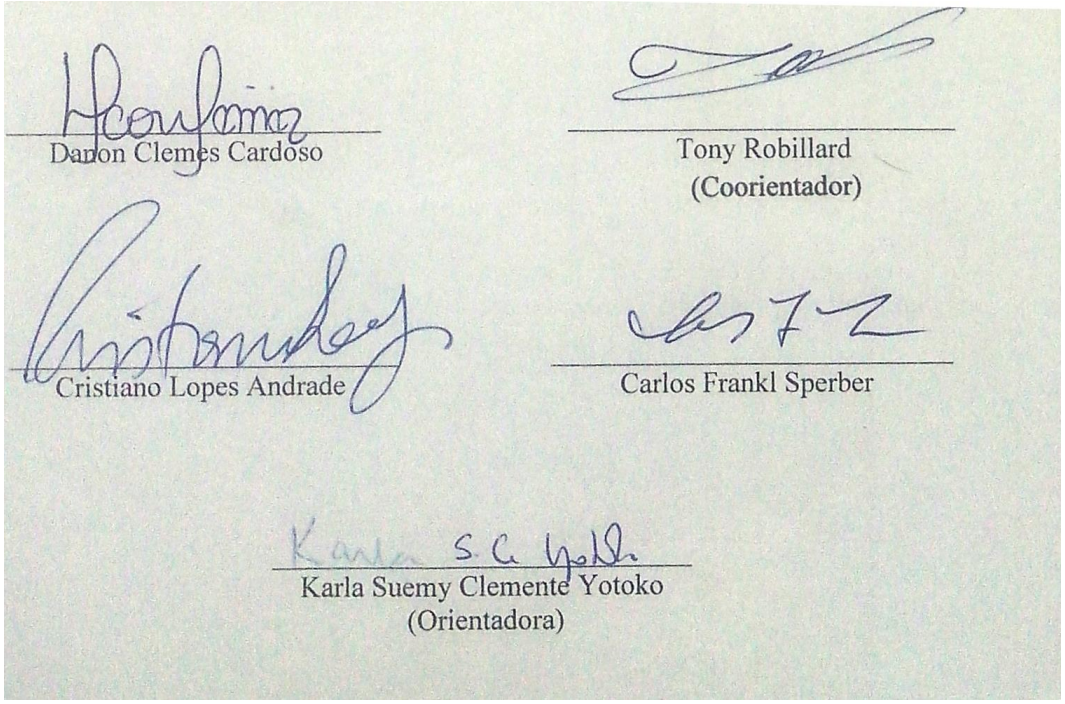

À minha Família

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RESUMO

VICENTE, Natállia Maria de Freitas, D.Sc., Universidade Federal de Viçosa, Dezembro de 2015. **Biogeografia, sistemática e evolução da comunicação acústica em grilos Eneopterinae**. Orientadora: Karla Suemy Clemente Yotoko. Coorientadores: Tony Robillard e Francisco de Assis Ganeo de Mello.

Eneopterinae é uma subfamília amplamente distribuída de grilos, com espécies descritas para a maioria das regiões tropicais do mundo. A subfamília foi recentemente alvo de estudos evolutivos e ecológicos uma vez que são os grilos conhecidos capazes de produzir chamados em alta freqüência. Dois grupos de Eneopterinae estão mostrando essa inovação no sistema de comunicação: Lebinthini, distribuídas na região do Pacífico e Eneopterini da região Neotropical. Essa tese visa dar respostas sobre a biogeografia e evolução da comunicação acústica dos grilos Eneopterinae na América do Sul. Nós também exploramos diferentes abordagens baseadas na morfologia e no DNA para tentar desvendar a complexa taxonomia deste grupo. No primeiro capítulo utilizamos fósseis do outgroup como pontos de calibração e análises biogeográficas para estimar a origem, quantas vezes Eneopterini colonizou a América do Sul e as rotas em direção a este continente. No segundo capítulo estudamos a bioacústica dos Eneopterinae Neotropicais. No terceiro capítulo, usamos abordagens baseadas no DNA e na morfolgia para explorar a diversidade de espécies em *Ligypterus*. Finalmente, no quarto capítulo, descrevemos um novo gênero de Eneopterinae, Gnominthus, dando ênfase à morfologia geral, as análises bioacústicas do chamado e a descrição do comportamento de acasalamento. Este estudo avança a compreensão dos processos biogeográficos mundiais que moldaram atuais padrões de distribuição. Nossos resultados mostram que a origen da subfamília é muito mais antiga do que o esperado e que a sua diversificação remonta o Creatáceo posterior (cerca de 76 Ma). Neste contexto, a colonização dos Neoptropicos teria ocorrido duas vezes de forma independente, primeiramente, a partir de uma origem antártica, resultante do desmembramento de uma fauna Gondwana, e mais tarde por uma recolonização do norte vindo do Sul da Ásia, provavelmente pelo cinturão Boreotropical durante o Eoceno. Nossos resultados indicam uma migração boreotropical para Eneopterinae, com interessante padrão de recolonização da América do Sul através do hemisfério Norte. A migração Boreotropical, é bem estudada em plantas, sendo uma importante explicação

biogeografia para este grupo, mas não é muito explorado em animais. No segundo capítulo, esclarecemos a evolução dos chamados em alta freqüência em Eneopterinae, mostrando um cenário de duas origens independentes para essa característica. No terceiro capítulo, os métodos de descoberta de espécies indicam uma diversidade consideravelmente maior no gênero Ligypterus, com o dobro da diversidade conhecida. E finalmente, no quarto capítulo, descrevemos um gênero endêmico de Eneopterinae proveniente de Papua Nova Guiné com um comportamento reprodutivo distinto e alta freqüência som de chamado. Acreditamos que as perspectivas que se abrem pela discussão sobre esta tese tem o potencial de oferecer base para novos estudos a respeito desta subfamília e grilos em geral. A exploração do uso de comunicação de alta frequência em outros grupos de grilos é urgente, dada a importância deste recurso nos processos evolutivos e ecológicos em Eneopterinae. O uso de método baseados em DNA podem esclarecer outros grupos de grilos com taxonomia complexa, revelando a verdadeira diversidade em Orthoptera.

ABSTRACT

VICENTE, Natállia Maria de Freitas, D.Sc., Universidade Federal de Viçosa, December, 2015. **Biogeography, systematics and evolution of acoustic communication in Eneopterinae crickets**. Advisor: Karla Suemy Clemente Yotoko. Co-advisors: Tony Robillard and Francisco de Assis Ganeo de Mello.

Eneopterinae is a widely distributed subfamily of crickets, with species described from most Tropical regions of the world. The subfamily has been recently target of evolutionary and ecological studies once they are the only crickets known to use high-frequency calling songs. Two groups of Eneopterinae are showing this innovation in the communication system: Lebinthini distributed in the Pacific region and Eneopterini from the Neotropics. Specifically, this thesis aims to give answers about the biogeography and evolution of acoustic communication of Eneopterinae crickets in South America. We also explore trough different approaches the complex taxonomy of this group. In the first chapter we used a dated phylogeny calibrated with fossils and biogeographical analyses to estimate the origin, how many times Eneopterini colonized South America and the routes toward and from this continent. In the second chapter, we studied the bioacoustics of the Neotropical genera of Eneopterinae crickets. In the third chapter we used DNA and morphology-based approaches to explore species diversity in the cryptic Eneopterini genus *Ligypterus.* And finally, in the fourth chapter we described a new genus of Eneopterinae, *Gnominthus*, focusing on general morphology, bioacoustical analyses of the calling song and the description of the mating behavior. This study advances the understanding of the global biogeographical processes that shaped current distribution patterns. Our dating results show that the subfamily is a Gondwanan group, far older than expected and that its diversification dates back to Late Creataceous (ca. 76 Ma). In this context the colonization of the Neoptropics would have occurred twice independently, very early from an Antarctic origin, resulting from the break-up of a Gondwanan fauna, and later by a northern recolonization coming from South-east Asia, likely related with a Holarctic Boreotropical distribution of the species during the Eocene. Our results indicate a boreotropical migration for Eneopterinae, with interesting patterns of recolonization of South America through the Northern hemisphere. Boreotropical migration, is well-studied in plants, for which it is an important

explanation for the biogeography, but is not very explored in animals. In the second chapter, we clarified the evolution of high-frequency songs in Neotropical Eneopterinae crickets, showing a scenario of two independent origins for this feature. In the third chapter, the species discovery methods indicated a considerably higher diversity in the cryptic genus *Ligypterus*, with probably twice more species than we currently know. And finaly in the fourth chapter we described an endemic Eneopterinae genus from Papua New Guinea with a distinct reproductive behavior and high-frequency calling songs. We believe that the perspectives now open by discussion on this thesis have the potential to offer base for further studies regarding this subfamily and crickets in general. The exploration of the use high-frequency communication in other cricket groups is urgent, given the importance of this feature in the evolution and ecological processes in Eneopterinae. The use of DNA based method may clarify other taxonomic complex crickets groups, unveiling the real diversity in Orthoptera.

GENERAL INTRODUCTION

Eneopterinae Saussure, 1874 (Gryllidae) is a widely distributed subfamily of crickets, with species described from most tropical regions of the world. The subfamily can be divided in five tribes: Eurepini Otte & Alexander, 1983 (Australia), Eneopterini Saussure, 1874 (South and Central America) Nisitrini Robillard, 2004 (Southeast Asia), Xenogryllini Robillard, 2004 (Africa, India, Japan, and Southern Asia) and Lebinthini Robillard, 2004 (Southwest Pacific Islands and Southeast Asia).

Eneopterinae has been recently target of evolutionary and ecological studies once they are the only cricket know capable of producing high-frequency calling songs, sometimes reaching more than 25.0 Khz (Robillard *et al.,* 2007). This is much more than what is expected for a cricket, which the dominant calling frequency varies between 2.0 and 8.0 KHz (Bennet-Clark 1989). Species in two tribes of Eneopterinae show this innovation in the communication system: Lebinthini, distributed in the Pacific region and Eneopterini from the Neotropics.

This innovation in acoustic communication may be influencing the evolution and ecological traits in Eneopterinae, since it may be associated with strategies to avoid predators and parasites, effectiveness of acoustic signal and favored pre-zigotic isolation (Robillard *et al.,* 2007). To determine the evolutionary history of the high-frequency calling, it is necessary to infer a proper phylogeny, which requires the identification of species. However, some groups of Eneopterinae present a considerable number of cryptic species (species morphologically indistinguishable morphologically *sensu* Bickford *et al.,* 2006; Pfenninger & Schwenk, 2007), making their taxonomy confusing.

Many gaps also still exist within the phylogeny of Eneopterini. Phylogenetic hypothesis based on morphological data suggested that the tribe constitutes a monophyletic group (Robillard & Desutter-Grandcolas, 2004). However, the most recent molecular phylogenetic hypothesis proposed for Eneopterinae (Nattier *et al.,* 2011) presented Eneopterini as a polyphyletic group. In morphological hypotheses, its monophyly is supported by few synapomorphies, but many morphological characteristics differ between *Eneoptera* Burmeiter, 1838 and the other two genera, *Ligypterus* Saussure, 1878and *Ponca* Hebard, 1928. In the molecular hypothesis presented in Nattier *et al.,* (2011), Eneopterini is a polyphyletic group and the high frequency calling emerged at least twice: in Lebinthini and whithin *Eneoptera* (*E. guyanensis* Chopard, 1931 and *E. nigripedis* Robillard, 2005). However, this study was focused on species of South-Pacific and New Caledonia, including only six Neotropical species (three *Eneoptera*, two *Ligypterus,* and one *Ponca*).

This thesis aims to give answers about the biogeography and evolution of acoustic communication of Eneopterinae crickets. We also explore trough different approaches the complex taxonomy of this group.

- In the first chapter we used a dated phylogeny calibrated with fossils and biogeographical analyses to address the following questions: (i) What is the ancestral distribution and origin of the subfamily nowadays distributed only in tropical areas, (ii) How many colonizations of Eneopterinae occurred in South America and (iii) what are the probable routes of colonization toward and from this particular area?

- In the second chapter we studied the bioacoustics of the Neotropical genera of Eneopterinae crickets and discuss the evolution of the only case of high-frequency calling known in crickets.
- In the third chapter we used DNA and morphology-based approaches to explore species diversity in *Ligypterus,* a Neotropical cricket genus containing a series of possibly cryptic species showing a wide array of morphological variation.
- The fourth chapter is a published study where we described *Gnominthus*, a new genus of Eneopterinae crickets from New Guinea Island (Papua New Guinea), which belongs to the tribe Lebinthini. Descriptions focus on general morphology, bioacoustical analyses of the calling song and the description of the mating behavior.

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CHAPTER ONE

Historical biogeography of Eneopterinae crickets: multiple colonizations of South America

Manuscript in preparation, to be submitted to *Journal of Biogeography*

Historical biogeography of Eneopterinae crickets: multiple colonizations of South America

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Keywords: Dispersal, boreotropical dispersion, outgroup calibration, disjunct distribution, South America

Abstract

Aim: We revisited the molecular phylogeny of the cricket subfamily Eneopterinae focusing on the Neoptropical lineage (tribe Eneopterini) in order to analyze the biogeography of the group, and their origin in the Neotropics.

Material and Methods: We used a dated phylogeny calibrated with fossils and biogeographical analyses made with Biogeobears to address the following questions: (i) what is the ancestral distribution and origin of the subfamily nowadays distributed only in tropical areas, (ii) how many colonizations of Eneopterinae occurred in South America and (iii) what are the probable routes of colonization toward and from this particular area?

Results: We showed that the previous phylogenetic reconstructions of the subfamily are stable and we confirmed that Eneopterini is polyphyletic. Our dating results showed that the subfamily is far older than expected based on previous results and that its diversification dates back to Late Creataceous (ca. 76 Ma). In this context the colonization of the Neoptropics would have occurred twice independently, very early from an Antarctic origin, resulting from the break-up of a Gondwanan fauna, and later by a northern recolonization coming from South-east Asia, likely related with a Holarctic Boreotropical distribution of the species during the Eocene.

Introduction

Eneopterinae (Gryllidae) is a widely distributed subfamily of crickets, with species described from most Tropical regions of the world. They have recently been extensively studied for their capacity to use high-frequency calling signals to communicate (Vicente *et al*., in prep).

While the phylogenetic relationships in the subfamily have been repeatedly studied to address questions related to the evolution of communication or local biogeographic patterns, the worldwide distribution of the clade was never analyzed using biogeographical tools. The first study investigating the phylogenetic relationships of the Eneopterinae was published by Robillard & Desutter-Grandcolas, 2004a. In this paper, based on a morphological data set (193 characters, 45 taxa), the authors defined five monophyletic tribes with distinct geographical origins: Eurepini Otte & Alexander, 1983 (Australia), Eneopterini Saussure, 1874 (South and Central America) Nisitrini Robillard, 2004 (Southeast Asia), Xenogryllini Robillard, 2004 (Africa, India, Japan, and Southern Asia) and Lebinthini Robillard, 2004 (Southwest Pacific Islands and Southeast Asia). The same pattern arose in the subsequent works that took into account morphological characters (Robillard & Desutter-Grandcolas, 2004b, Robillard & Desutter-Grandcolas, 2005; Robillard, 2006; Desutter-Grandcolas *et al.,* 2010; Robillard & Desutter-Grandcolas, 2011).

Clearly, two main categories of hypotheses may explain such a worldwide distribution: 1. Vicariance caused by the continental drift, and/or 2. Dispersal, that occurred after the separation of the continents. In the first scenario the origin of the lineages must occur before the geological events leading to isolation. In the second scenario, dispersal, survival and establishment of individuals in far areas

are rare events in the case of transoceanic disjunctions (Crisp *et al.,* 2011). However, even being rare, the long distance dispersal should not be discarded as an explanation for nowadays distribution patterns, in particular dispersal between islands (Gillespie *et al.,* 2012).

In 2006 Robillard & Desutter-Grandcollas used molecular markers (16S rRNA, 12S rRNA, Cytochrome b, 18S rRNA) for the first time, together with morphological characters, to reconstruct the phylogenetic relationship of the subfamily. Intriguingly, Eneopterini appeared as a polyphyletic group in most of the analyses: "*Ponca* + *Ligypterus"* grouped as a monophyletic group, while *Eneoptera* appeared as the sister group of all tribes, except for the Australian group, Euripini.

The most recent molecular hypothesis (Nattier *et al.,* 2011) also suggests that the Eneopterini may constitute two different lineages, in a study based on molecular markers only, but focusing on species of the South Pacific and New Caledonia, including only six Neotropical species (three *Eneoptera*, two *Ligypterus,* and one *Ponca*). The molecular dating made in this study was intended to test whether the age of the clades distributed in New Caledonia could be older or not than the emergence of the main island of this archipelago. In this context, the authors defined a conservative strategy to used geological events external to the region of interest as calibration points, and only one uncertain fossil, described exclusively from a wing print.

Gondwana break up played an important role for the current biogeography pattern in animals with disjunct distribution like Eneopterinae (Sanmartin & Ronquist 2004). In Nattier *et al.,* 2011, the origin age of Eneopterinae is estimated at 45 Ma, which would discard the hypotheses of vicariance and the breakup of Gondwana modeling the present biogeography of the subfamily. At this time,

during Eocene, all the continents were already separated. Although efficient to test the biogeography hypothesis at the scale of New Caledonia, the calibration strategy used in Nattier *et al*., (2011) is likely to have underestimated the dates at the deeper nodes of the tree (Hipsley & Muller, 2014), and the age of 45 Ma found for Eneopterinae, which would immediately discard any vicariance hypothesis, should be considered with caution as far as questions about the whole clade's distribution are concerned.

A review of the morphological traits used to base the first phylogenies shows that few synamopomorphies actually support the monophyly of the Neotropical species, and many morphological characteristics actually differ between *Eneoptera* and the "*Ligypterus – Ponca"* clade. Therefore, despite the time and quantity of studies concerning phylogenetic relationship of the subfamily, many gaps still exist within the Eneopterini. Also in terms of bioacoustics, all the genera within Eneopterini use high-frequency songs to communicate (Robillard *et al.,* 2015), which, together with the geographic distribution, would be a good reason to find them in a monophyletic group, despite many differences between them.

In this work, we revisited the phylogeny of Eneopterinae by expanding the gene sampling and the taxonomic sample, in particular for Neotropical genera, *Eneoptera*, *Ponca* and *Ligypterus.* We performed a new molecular clock dating of the subfamily, based on a more complete set of cricket fossils and analyses in order to document the biogeographical history of the subfamily, and to determine if the worldwide distribution results from vicariance and/or dispersal events. In particular, we aimed to confirm if Eneopterinae colonized South America twice and what are the probable routes of colonization toward and from this particular area.

Material and method

Character and taxon sampling

The ingroup dataset comprises 94 specimens corresponding to 62 species of Eneopterinae, representing all tribes and genera of this subfamily.

We sampled several populations of South American species across Brazil using material collected during field expeditions of the Brazilian Orthoptera Biota research network that has been collecting samples throughout Brazil since 2011. These were collected by pitfall using ethanol as killing solution (Szinwelski *et al.,* 2012) that conserves the DNA. We also obtained neotropical Eneopterinae from the Orthoptera's collection of Universidade Estadual Paulisa , Botucatu, São Paulo and from recent collections made in French Guiana (Our Planet Reviewed Expedition, MNHN/Pro Natura International) (F. Legendre and S. Hugel, MNHN).

For outgroup comparison we used an approach based on the availability and quality of fossils in Grylloidea provided by the database Orthoptera Species File Online. We selected outgroup species from a recently published phylogeny of crickets (Marquier *et al.,* in press) for which molecular data was available for six of our studied markers (18S, 28S, H3, Cytb, 16S, 12S), and additional data were obtained for some of these species for the gene CO1. For each fossil species preselected for calibrating the molecular tree, we sampled two or three species of the corresponding clade in the cricket phylogeny. Using this strategy, we used 21 outgroup species encompassing the Gryllotalpidae, Mogoplistidae, Trigoniididae, Phalangopsidae, Gryllidae, plus two Acrididae species (Caelifera) to serve as more external outgroups. The complete list of ingroup and outgroup specimens

and accession numbers are presented in Table S1 (Appendix S1 - Supplementary material).

DNA extraction, amplification and sequencing

The molecular phylogeny was reconstructed using a dataset composed of 3330 base pairs encompassing seven molecular markers, three nuclear and four mitochondrial. The nuclear markers consist of fragments of 18S ribosomal RNA (~650 bp), histone H3 (~330 bp), 28S ribosomal RNA (400 bp). The mitochondrial markers are fragments of the 16S ribosomal RNA (~500 bp), 12S ribosomal RNA (~400 bp), cytochrome oxidase subunit I (~650 bp) and cytochrome b (~400 bp) (Table S2 Appendix S2– Supplementary Material). In addition to the sequences generated for this study, we obtained sequences available on GenBank from previously studies on Eneopterinae.

The DNA extraction of fresh specimens followed the extraction protocol of Waldschmidt *et al.,* (1997), with slight modifications. For samples of the collection, DNA extraction was performed using the QIAamp DNA MICROKIT (QIAGEN) following the manufacturer's instructions. The amplification chain reaction (PCR) consists of an initial denaturation step at 94 °C for 3 min for separation the DNA strand. Subsequently, 30 to 40 cycles of amplification (denaturing at 94 °C for 30s, annealing at 48-58 °C for 40s) and a final step at 72 °C for 5 minutes. Some samples of mitochondrial genes (CytB and CO1) had unspecific bands. We purified de DNA fragment from the agarose gel using the PureLink ® Quick Gel Extraction Kit. The fragments were sent to sequencing in Macrogen (Korea) and Eurofins (France). The sequence's quality were analysed under Sequencher v5.1.

Alignments and phylogenetic analyses

We searched for sequences in Genbank for the 28S and CytB of the specimens belonging to the outgroup species *Locusta migratoria* and *Schistocerca gregaria.* To align the sequences we used the software MUSCLE (Edgar, 2004) with default parameters, implemented in MEGA 6.0 (Tamura *et al.,* 2013). With MEGA 6.0 we also checked if the protein-coding genes were congruent with codon reading frame. We conducted preliminary maximum likelihood (ML) + rapid bootstrap (200 reps) analyses in the software RAxML v.7.4.2. run with raxmlGUI v. 1.3.1 (Stamatakis, 2006; Silvestro and Michalak, 2012) with each gene separately to check for contaminations and artifacts. With the software Sequence Matrix 1.7.8 (Vaidya *et al.,* 2011) we concatenate our sequences and used Partition Finder v 1.1.1 (Lanfear *et al.,* 2012) with Bayesian Information Criterion (BIC) (Schwarz, 1978) to infer the best partitioning scheme and substitution model for RAxML, MrBayes and BEAST. For that, the coding genes were separated according to codon position. Four partition were selected for the three analysis. We then performed an ML search with 100 replicates plus 1000 non-parametric bootstrap in RaXML. In MrBayes, eigth Markov chains were run simultaneously for 50 million generations, sampling every 1000 generations to ensure independence of samples. We used a conservative burn-in-period of 12.5 million generations per run. A clade with a PP value higher than 0.95 was considered as well supported. Trees were finally visualized with FigTree v.1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Fossil calibrations and molecular dating analysis

Dating analyses were performed using the Bayesian relaxed clock (BRC) approach implemented in BEAST v1.8.1 (Drummond & Rambaut 2002-2015).

After an exhaustive search in the "Orthoptera Specie File" database, we found no reliable fossils for a direct calibration within our study group. The only fossil records known assigned to the Eneopterinae are the 96 million years old *Proecanthus anatolicus* Sharov, 1968 and the 122 million years old *Brontogryllus excelsus* Martins-Neto, 1991. Both fossils are, however, based on the incomplete impressions of the tegmen, and cannot be clearly assigned to this subfamily. The problems associated to geological and secondary calibrations are well known (Hipsley & Muller, 2014). To avoid that, we choose to use a wide-outgroup approach to calibrate our dating analyses (Hedges & Kumar, 2004; Strijk *et al.,* 2012) based on fossils that, according to the description, could be reliably addressed to a stem group. Sauquet *et al.,* (2012) found no drastic influence in analysis using only ingroup or only outgroup calibration and suggested that in the absence of a suitable age constraints for the ingroup, outgroup calibration might be an appropriate solution. Therefore, using date of external fossil that we can reliably assign to a node within a thoroughly selected set of outgroup species, we expect to more precisely estimate the ages of divergence for the ingroup.

We used a conservative approach, based on apomorphy, to asses fossil relationships with extant taxa based on the latest molecular phylogeny published for the superfamily Grylloidea (Chintauan-Marquier in press). First, we created a complete dataset based on the fossils published in "Orthoptera Specie File" database (139 described fossils). We then pre-selected the oldest fossils of each family based on a detailed analysis of their description. We chose those fossils that presented apomorphies that clearly place them to the stem of a group. In those cases that the oldest ones had a bad description we came back to the dataset and chose the next according to the age. In particular, for Gryllidae, the diagnostic characters are, until today, confuse. The choice of †*Araripegryllus camposae*

Martins-Neto was based in the presence of the following features: venation wing pattern, prominent eyes, and posterior tibia with at least 3 apical spurs. An extensive material was collected by the author for the description of this specie (including wings, legs and complete body impression) showing several evidence that it is a Gryllidae. Nevertheless these diagnostic characters are too general, and the 2 crossveins presented in the mirror are an important character in Phalangopsinae, primitive Baissogryllidae and early Cretaceous subfamilies of possible Gryllidae (Gorochov, 2012). In this manner, we chose to place him in a more basal node.

We pre-selected fossils belonging to Gryllotalpidae, Mogoplistidae, Trigonidiidae, Gryllidae, and Subfamily Luzarinae (Gryllidae) (Table 1). Up to four primary fossil calibrations were enforced to provide a more precise estimation of divergence times. A first set of analyses relied on two fossil constraints. For the first constraint we used the age of †*Palaeoscapteriscops cretacea* Martins-Neto, the oldest known mole cricket fossil (Gryllotalpidae), to set a constraint on the stem node of sampled mole crickets. This fossil (an exoskeleton; type specimen 'GP/1T 1682') was found in Aptian lacustrine belonging to the Crato Formation of Brazil (age range: 122.46 to 112.6 Ma). Second, we relied on †*Baltonemonius fossilis* Gorochov, the oldest known fossil of Trigonidiidae to set a constraint on the stem node of sampled trigonidiids. This fossil (an amber-preserved nymph; type specimen 'ZIN Balt. 3') was found in Priabonian terrestrial Baltic amber in the Russian Federation (age range: 37.2 to 33.9 Ma). A second set of analyses also included two additional (and more recent) fossils. We used two fossils found in Burdigalian/Langhian terrestrial amber in the Dominican Republic (age range: 20.43 to 13.65 Ma): *(i)* the oldest known fossil of spider cricket (Phalangopsidae), †*Araneagryllus dylani* Head (an amber-preserved

exoskeleton; type specimen 'AMNH DR-12-32'), was used to set a constraint on the stem node of sampled spider crickets; *(ii)* the oldest known fossil of anomalous crickets (Gryllidae: Pentacentrinae), †*Proanaxipha madgesuttonae* Head & Penney, (an amber-preserved exoskeleton; type specimen 'NHM II 3048'), was used to set a constraint on the stem node of sampled anomalous crickets. To limit the risk of age overestimations we also used as an upper bound the minimum age associated with the stem age of Gryllidae (235.0 Ma), which corresponds to the oldest known occurrence of a member of Grylloidea (Heads & Leuzinger, 2011). All fossil constraints were further enforced using either a uniform or an exponential statistical distribution (Yang & Rannala, 2006). For the calibrations relying on exponential distributions we used the following settings to set 95% of the prior distribution: *(i) mean* = 33.42; *offset* = 111.75 for the fossil from the Aptian lacustrine, *(ii) mean* = 53.12; *offset* = 39.06 for the fossil from the Baltic amber formation, and *(iii) mean* = 60.45; *offset* = 11.965 for the fossils from the Dominican amber formation. BEAST analyses were further implemented with a birth-death tree speciation prior to account for the fact that our trees describe inter-specific relationships. To limit the number of parameters to estimate we also used a guide tree that corresponds to the topology inferred with MrBayes.

Bayesian relaxed clock analyses were carried out using either uncorrelated lognormal relaxed (UCLN) or random local (RLC; Dornburg *et al.,* 2012) clocks. As a result we set up a total of eight distinct calibration procedures for the BRC analyses (UCLN vs. RLC; 2 or 4 fossil constraints; uniform or exponential distributions). Four distinct relaxed clocks were specified based on the result of a Partition Finder analysis (with the *beast* option). For each calibration procedure, two distinct runs were carried out with 50 million

generations and trees sampled every 5,000 generations (10,000 trees were sampled for each run). Based on the result BEAST xml files were modified to implement the path-sampling procedure for B_F estimation following the recommendations of Baele *et al.,* (2013). We used a conservative burn-in-period of 12.5 million generations per run. Post burn-in trees from the two distinct runs (7,500 trees for each run) were further combined using the LogCombiner module of BEAST. Convergence of runs was assessed graphically under Tracer v1.5 (available at http//tree.bio.ed.ac.uk/software/tracer/) and by examining the effective sample size (ESS) of parameters.

Historical biogeography

Together with the configuration of the continents, geography and climate of the landmasses also changed, affecting the distribution of the species during time. We divide our biogeographic model in different time slices in order to reflect the continental changes that occurred since the breakup of Gondwana.

Historical biogeography analyses were carried out using the R package BioGeoBEARS (BioGeography with Bayesian (and likelihood) Evolutionary Analysis of RangeS (Matzke, 2014). This package relies on the LAGRANGE (Ree *et al.,* 2005; Ree and Smith, 2008) dispersal extinction cladogenesis (DEC) model. It also implements a new model called DEC +J model which accounts for founder event speciation (Matzke, 2014). Here we carried out distinct analyses for the DEC and DEC +J models. As a guide tree we used the dated phylogeny corresponding to the best-fit calibration procedure. This tree was further pruned to only include Eneopterinae, in order to avoid potential biases resulting from the fact that outgroup taxa were sparsely sampled. We defined ten geographical

areas for the BioGeoBEARS analysis after considering the evidence available for historical relationships between relevant geographic areas (Sanmartín *et al.,* 2001; Sanmartín and Ronquist, 2004) and the distribution of *Spermophagus* taxa. These biogeographic regions were as follows:

(i) Africa.

(ii) Australia.

(iii) Central America: Costa Rica, Nicaragua and Panama (this area arose during the Neogene (23.3 to 2.58 Ma) as a result of the collision between the Panama microplate and the South American plate).

(iv) India: India, including Nepal and Sri Lanka.

(v) New Caledonia. This area began to separate from Australia 80 Ma. During this time, New Caledonia was submersed, emerging only in the Oligocene (37 Ma) (Grandcolas *et al.,* 2008).

(vi) New Guinea: New Guinea, including Solomon Island.

(vii) Palearctic.

(viii) South America.

(ix) Southeast Asia: Southeast Asia, including Borneo, Java, the Malaysian Peninsula, the Philippines and Sumatra. Southeast Asia was formed by the accretion of terranes that rifted away from Gondwana. Part of the Malay Peninsula and Borneo were already formed in the Cretaceous. The remaining insular terranes (Sumatra, the rest of Borneo, Celebes, the Inner Banda Arc, etc.) were formed as a result of the collision of the Australian Plate with the Eurasian Plate during the Cenozoic).

(x) Southwest Pacific. This area includes the Melanesian archipelagos formed during the Late Eocene/Oligocene: Fiji (33.9 to 38 Ma – late Eocene), Samoa (23 Ma), Tonga (45 Ma) and Vanuatu (25 Ma).

Following the views of several authors (Hines, 2008; Mansion *et al.,* 2008; Nylander *et al.,* 2008), taxa with marginal distribution in an area were not assigned to it. Species ranges were coded by presence–absence and a maximum number of two areas was set for both DEC and DEC +J analyses.

Finally, to account for major periods of geological rearrangements, we used time-stratified biogeographical models, with three distinct time slices. The first time slice (*t1)* runs from 65.0 to 80.0 Ma. The second time slice (*t2)* runs from 30.0 to 65.0 Ma. The last time slice (*t3)* runs from 0.0 to 30.0 Ma and corresponds to the highest level of connectivity between all considered geographic areas. Central America, New Caledonia and Southwest Pacific were not present in the time of origin of Eneopterinae. Therefore, they were excluded from the time slices that correspond to periods before the formation of these areas. For each time slice we provide a probabilities of dispersion matrix (Appendix S3 – Supplementary Information), constructed according to the geographical connectivity between areas. To measure this connectivity we also consider land bridges between the landmasses that could favour biota exchange (Kerguelen Plateau). The rate of dispersion was scaled according to the availability of area connection through time. For example, for two areas that were connected in a time slice, with no barriers between them, we used a factor of 1.0, and for areas not connected in a given time slice we gave a factor of 0.01 to reflect the low probability of dispersion. The factors were as follows (modified from Condamine *et al.,* 2013): *(i)* adjacent areas: 1.0; *(ii)* areas

separated by a small barrier: 0.7; *(iii)* dispersal between two areas separated by one area: 0.3; *(iv)* long distance dispersal: 0.01; *(v)* no dispersal: 0. After running both analyses, we used likelihood ratio tests (LRT) to sort between the two competing models (DEC and DEC +J), as recommended by Matzke (2014).

Results

Phylogenetic analyses

Given that the most recent molecular phylogeny dealing with Eneopterinae (Nattier *et al.,* 2011) sampled four markers (3 mitochondrial and 1 nuclear) and 43 species, this study significantly increased the sampling of genes and species for the subfamily. We improved the sampling with specimens of different localities, particularly for South American species, which were previously poorly sampled.

The separated analyses of each marker showed no major conflict of topology. On the combined dataset, the Bayesian and ML analyses yielded largely congruent topologies, which recover Eneopterinae as monophyletic with high support (Bootstrap (BS) in ML analysis = 96; PP =1).

With exception of Eneopterini, all the existing tribes are monophyletic and can be matched to supported lineages in our analysis. Basally, the subfamily is separated by two groups, one encompassing the Australian Eurepini (BS: 100; PP: 1) with the genera *Salmanites, Eurepa, Myara, Arilpa* and *Eurepella* (clade I) and the other consists of all remaining tribes. Clade II comprise *Eneoptera,* one genus of the polyphyletic Eneopterini, (BS: 100; PP: 1). In the clade III are gathered *Paranisitra* and *Nisitrus,* the two genera of the tribe Nisitrini (BS: 100; PP: 1). The Xenogryllini with *Pseudolebinthus* and *Xenogryllus* form the clade IV (BS: 100; PP: 1). Clade V consists of two separate tribes: the speciose Lebinthini and

the other two genera of Eneopterini, *Ligypterus* and *Ponca* (BS: 88; PP: 1). In this clade we also find *Swezwilderia* a genus with uncertain position in Eneopterinae. In Lebinthini the genus *Lebinthus* is paraphyletic. The other generic relationships are well defined, with all genera being monophyletic.

Time of divergence

For all dating analyses ESS $\geq$ 200 were obtained for all parameters. Out of the eight calibrations, the calibration procedure relying on two fossil constraints, an uncorrelated lognormal clock and exponential distributions has the best harmonic mean (-66783.39) and is significantly recovered as the best-fit calibration procedure in all but one B_F comparisons (Table 2).

The estimation of divergence time using 2 fossil calibration suggested that Eneopterinae diverged from other Gryllidae ca. 80 Ma (95% highest posterior density (HPD) confidence interval: 66-101 Ma) (Figure 1). The crown group Eneopterinae originated during late cretaceous ca. 76 Ma (95% HPD confidence interval: 63-96 Ma). The first split within the Australian group Eurepini occurred ca. 58 Ma (95% HPD confidence interval: 46-74 Ma) when *Salmanites* diverged from *Arilpa*, *Eurepa*, *Myara* and *Eurepella*. Within the polyphyletic Eneopterini, the most primitive *Eneoptera* lineage branched of first during late cretaceous ca. 70 Ma (95% HPD confidence interval: 58-90 Ma). The crown group began to diverge in early Miocene at 22 Ma (95% HPD confidence interval: 16-30 Ma). The split of *E. guyanensis* and *E. nigripedis* occurred ca. 13 Ma (95% HPD confidence interval: 8-19 Ma). *Eneoptera gracilis* and *E. surinamensis* separate ca. 12 Ma (95% HPD confidence interval: 7-19 Ma). The mean stem age of Nisitrini (SE Asia) was estimated at 67 Ma (95% HPD confidence interval: 55-86

Ma) and a mean crown age of 51 (95% HPD confidence interval: 40-67 Ma). The Xenogryllini diverged from the other Eneopterinae ca. 59 Ma (95% HPD confidence interval: 49-76 Ma). The tribe two genera *Pseudolebinthus* and *Xenogryllus* separated at 52 Ma (95% HPD confidence interval: 41-67 Ma). The mean stem age of *Swezwilderia* from southwest pacific islands was estimated at 48 Ma (95% HPD confidence interval: 38-62 Ma). Within the neotropical lineage, the ancestor of *Ponca* and *Ligypterus* appeared 40 Ma (95% HPD confidence interval: 30-52 Ma). *Ligypterus* first began diversifying in Miocene ca. 12 Ma (95% HPD confidence interval: 9-17 Ma). *L. fuscus* and *L. sp* (Manaus) separate ca. 9 Ma (95% HPD confidence interval: 6-13 Ma). The split of *L. fuscus* and *L. pernambucensis* occurred ca. 7 Ma (95% HPD confidence interval: 4-11 Ma). The mean stem age of Lebinthini was estimated at 50 Ma (95% HPD confidence interval: 44-69 Ma). The crown group of *Cardiodactylus* began diversify at 42 Ma (95% HPD confidence interval: 33-55 Ma). For the polyphyletic *Lebinthus* the first group encompassing the species from Southeast Asia and Palearctic area seems to have diverged from the other Lebinthini at 43 Ma (95% HPD confidence interval: 35-56 Ma) while the second lineage, encompassing species from Southwest pacific, Papua New Guinea and New Caledonia diverged at 40 Ma (95% HPD confidence interval: 33-52 Ma). *Centuriarius* had its origin at Oligocene ca. 31 Ma (95% HPD confidence interval: 24-42 Ma) after splits with *Gnominthus. Agnotecous* an endemic genus from New Caledonia has the mean stem age estimated at 31 Ma (95% HPD confidence interval: 24-40 Ma) with subsequent divergence of crown group dated at 24 Ma (95% HPD confidence interval: 18-31 Ma).

Biogeographical analyses

Model selections through LRT significantly support the DEC+J model (L = -107.44) over the DEC model (L = -116.09) as the best-fit model for the BioGeoBEARS historical biogeography analyses. The corresponding inference of ancestral areas evolution (Fig. 2) suggests an origin in Australia for Eneopterinae during the Late Cretaceous. Subsequently one lineage underwent diversification in Australia (tribe Eurepini) whereas the second one colonized the Western hemisphere through South America. An interesting pattern is then suggested, involving a colonization of the Old World (Southeast Asia) from the Western hemisphere *ca*. 70 Ma. This dispersal event was followed in the Paleocene by a colonization of India. During the Oligocene, several lineages also reached the New Guinea and the Southwest Pacific from Southeast Asia. One lineage (now encompassing the genera *Ligypterus* and *Ponca*) also went back to the Western hemisphere at the same period. Colonization of New Caledonia occurred more recently, shortly after the re-emergence of the archipelago. Analyses with a DEC model also recover a very similar pattern, and mostly differs by the fact that it infers several vicariance events (contrary to the result of the DEC+J analysis).

Discussion

Phylogenetic and systematic account

This study presents the most complete molecular phylogeny of the world distributed Eneopterinae. The obtained phylogenetic relationship among Eneopterinae lineages match the topology of Nattier *et al.,* (2011). We expanded this latest molecular phylogeny adding four genera and 15 species to the ingroup and adding more molecular markers from both nuclear and mitochondrial origins.

Our study confirms the polyphyly obtained by Nattier *et al.,* (2011) for Eneopterini, which was not found when morphological data were used for phylogenetic reconstruction (Robillard & Desutter-Grandcolas 2004a, 2006a). Our increase of Eneopterini populations clearly confirms the polyphyly of this tribe.

The branch leading to the crown of *Eneoptera* diverges very early in all analyses as the sister group of all other non-Australian Eneopterinae. The two other Neotropical genera, *Ponca* and *Ligypterus*, are more closely related to the tribe Lebinthini, which is corroborated further (Robillard *et al.,* 2015; Vicente *et al*., in prep) by the acoustic characteristics shared by these clades. *Ponca* and *Ligypterus* are more similar to Lebinthini than to *Eneoptera*. In terms of morphology, the grouping of these genera under Eneopterini appears to be a reconstruction artifact due to weak synapomorphies about details of female venation and male genitalia. The tribe Eneopterini should thus be restricted to the genus *Eneoptera* until further notice. According to the recent molecular phylogenies, the genera *Ligypterus* and *Ponca* are close to the Lebinthini and are therefore transferred to this tribe. This relationship is confirmed by strong, repeated molecular results, and independently by acoustic evidences (Robillard *et al.,* 2015). As a consequence, the clade "*Ponca +Ligypterus"* should be considered as a Neotropical branch of the Lebinthini.

Our study allows confirming the positions of the genera *Centuriarus* and *Gnominthus,* recently described and never included in a phylogeny until now, within the tribe *Lebinthini.* These two genera form a clade located between the paraphyletic *Lebinthus* and the clade "*Agnotecous + Pixibinthus"*, which confirms the current paraphyletic situation of *Lebinthus* (Robillard *et al.,* in prep.).

The genus *Swezwilderia*, previously considered as Eneopterinae *insertae sedis* (Robillard & Desutter-Grandcolas, 2008), is confirmed within the tribe Lebinthini. The position of *Swezwilderia* was previously uncertain, based on morphological phylogeny only (Robillard & Desutter-Grandcolas, 2004a; Robillard 2006), but the recent molecular studies confirm that the genus belongs to the Lebinthini tribe (Nattier *et al.,* 2011; Marquier *et al.,* 2015, this study), close to the clade "*Ponca* + *Ligypterus*", although this relationship is yet poorly supported.

Historical biogeography of Eneopterinae

This study is the first attempt to reconstruct a biogeographical scenario for a clade of crickets with a world-wide distribution using modern phylogenetic methods for historical biography. Our results suggest that both vicariance and dispersal promoted diversification of the group under study.

Our dating analysis suggests that the radiation of Eneopterinae began in Late-Mid Cretaceous, ca. 76 Ma (95% HPD confidence interval: 63-96 Ma). This result is much older than the 46 Ma found by Nattier *et al.,* (2011), which underestimated the age of the clade by using recent geographical calibration points.

As a consequence of the older age of the subfamily, Eneopterinae would constitute a Gondwanan group. If we take into account the confidence intervals, or just considering the average age of 76 Ma, all or most Gondwana landmasses were still connected at the origin of the ancestral Eneopterinae. Africa and South America were connected until ca. 95 Ma (Sanmartín & Ronquist, 2004, Sereno *et al.,* 2015). The Drake Passage was an important dispersal route between South

America-Antarctica-Australia-New Guinea at this time (Sanmartín & Ronquist, 2004). An alternative route between India and Australia was possible through the Kerguelen Plateau during the mid-Cretaceous and possibly for some time beyond (Sanmartín & Ronquist, 2004, Hay 1999, Ali & Aitchison 2008). Therefore, it would be impossible to predict the ancestral area of Eneopterinae without a formal biogeographical analysis. Our BioGeoBEARS historical biogeography analyses suggest that the subfamily originated in Australia during the Late Cretaceous, which was not yet disjoint from Antarctica at this period. Subsequently one lineage underwent diversification in Australia (Eurepini) whereas the second one colonized South America (genus *Eneoptera*, nowadaways distributed in the north of South America). The Western hemisphere (Southeast Asia, tribe Nisitrini) was thereafter colonized during Early Paleocene, thought a northern route of colonization, either through a trans-atlantic pathway, or, less through the Bering Strait, even if the latter is less likely, as suggested by Sanmartin *et al.,* (2001): "...These analyses show that trans-Atlantic distributions were common in the Early-Mid Tertiary (70-20 Myr), whereas trans- Beringian distributions were rare in that period".

It constitutes a classical case of parallel Boreotropical migration from north to south (Sanmartin *et al.,* 2001). The second colonization of South-America by Eneopterinae would thus follow the same route as the previous colonization of Asia from South-America, but in the other sense. However, considering that most of the Holarctic region had a tropical climate in this period, the recolonization of South-America by *Ligypterus-Ponca* from the Boreotropical region may have been greatly facilitated.

In parallel, the dispersal of *Swezwilderia* from Southeast Asia toward islands of the Southwest Pacific (Fiji, Samoa) was probably due to ocean drift,

since these islands were not connected to landmasses and were formed only during the Late Eocene – Oligocene (Neall & Trewick 2008). The biodiversity of islands that were not formed by the fragmentation of continental landmasses depends fundamentally on oceanic dispersal. According to Gillespie *et al.,* (2012), the major vectors for such long distance dispersal in the Pacific are wind, ocean drift and birds. The dispersal also follows general patterns like oceanic currents, bird's migratory route and wind patterns (Cowie & Holland 2006). We believe that wind patterns, storms and oceanic drift had played important role in dispersion of crickets in the pacific.

New Caledonia has a complex geological history. Together with New Zealand, New Caledonia separated from the Gondwana block very early during the late Cretaceous ca. 80 Ma (Sanmartín & Ronquist, 2004). From the end of the Cretaceous to the mid-Cenozoic, New Caledonia was however under water, emerging only 37 Ma (Grandcolas *et al.,* 2008, Pillon 2012). According to our study, New Guinea was an important source area of dispersal for New Caledonia, as previously suggested for other groups (Sanmartín & Ronquist 2004, Pillon 2012, Swenson *et al.,* 2015). According to our results, the ancestor of the endemic genera *Agnotecous* and *Pixibinthus* arrived in New Caledonia ca. 31 Ma, diverging from New Guinea clade *Centuriarus-Gnominthus* and giving rise to a highly diverse group.

Multiple colonizations of Eneopterinae in South America but lack of diversity

Eneopterinae colonized South America twice independently. First, the early divergence of the ancestor of *Eneoptera* (70 Ma) suggests a history of dispersal from Australia (Antarctica) to South America through the Drake

Passage, despite the fact that the genus is only distributed in the northern part of South America. The subsequent history of this lineage has probably been followed by multiple extinctions, as suggested by the long branch leading to the crown of the genus, or by contraction of its distribution due to climatic factors which could explain the absence of the genus or its relatives in the southern part of South-America. We discard the hypothesis that the position of *Eneoptera* could be an artifact of long-branch attraction, as it is recovered both in ML and Bayesian analyses, both with the concatenated dataset and separated gene dataset.

The Northern Andean uplift (23 Ma) marks the beginning of divergence within *Eneoptera*. This geological event played an important role in the landscape and biota of the entire part of northern South America, including Amazonia (Hoorn *et al.,* 2010). Subsequent divergences within the group correspond to two important moments: (I) the Andean uplift and resulting influx of sediments into the Amazonic basin created different aquatic environments in Amazonian landscape. The Acre phase occurred ca. 11 Ma and resembled the present-day Brazilian biome Pantanal (Hoorn *et al.,* 2010). The pollen records suggest the presence of rainforest combined with grasslands and/or floating meadows (Hoorn 2010). This new environments contributed to increase the local diversity, but subsequent decline of this wetland isolated populations and originated diversification in aquatic invertebrates (Wesselingh *et al.,* 2001), reptiles (Salas-Gismondi *et al.,* 2015), mammals (Matauschek 2010) and amphibians (Fouquet *et al.,* 2014). (II) The mid-Miocene climatic optimum was an episode between 17 and 15 Ma marked by a wet period followed by abrupt changes of temperature. These rapid environmental changes may have led to forest expansion and contraction, followed by isolation of forest-adapted species (Hinojosa 2005,

Fouquet *et al.,* 2014). These two events could markedly affected *Eneoptera* lineages.

Within *Eneoptera* the distribution pattern is contrastant, with species having restricted habitats (*E. gracilis, E. guyanensis* and *E. nigripedis*) and *E. surinamensis* characterized by a wide distribution (Robillard & Desutter-Grandcolas 2005). The habitat fragmentation and new environmental exposure could result in sister taxa that occupy contrasting habitats (Damasceno *et al.,* 2014). The expansion and retraction of forests induced by Andean uplift and the mid-Cretaceous optimum probably exposed the species of *Eneoptera* to different habitat conditions. This process could have resulted in the selection of populations "pre-adapted" to this new open environment, leading to the divergence of a clade adapted to completely different, open habitat. Therefore, this could be the explanation for the contrast distribution pattern observed among the species of *Eneoptera*.

The second colonization of Eneopterinae in South America occurred around 48 Ma (95% HPD confidence interval: 38-62 Ma) with the divergence of the clade *Swezwilderia*-(*Ponca-Ligypterus*) from the rest of the Lebinthini. Once in South America, the divergence between *Ponca* and *Ligypterus* match the uplift of central Andes (Hoorn *et al.,* 2010). The beginning of divergence within *Ligypterus* can also be a result of abrupt changes in temperature subsequent to the Mid Miocece climatic optimum. Nowadays this genus is distributed mainly in the Southeastern coastal Brazil, but a few species and populations occurs in the Amazon forest, in French Guiana and near Manaus. The coastal distribution of the genus appears to be limited by the major basin in Brazil (Vicente *et al.,* in prep) (Doce, Jequitinhinha and São Francisco), suggesting a riverine-barrier hypothesis (Moritz *et al.,* 2000). The genus also shows biogeographic pattern that indicates

past connections between Atlantic Forest and Amazon Basin (Vicente *et al.,* in prep).

Conclusion

This study advances the understanding of the global biogeographical processes that shaped current distribution patterns of Eneopterinae. We provide a world-wide dated biogeographical model for Eneopterinae crickets. Overall, the results of our study fit well with general predictions proposed for the evolution of groups with disjunct distribution and gondwana origin (Sanmartin & Ronquist, 2004). Our results indicate a boreotropical migration for Eneopterinae, with an interesting pattern of recolonization of South America through the Northern hemisphere. Boreotropical migration, is well-studied in plants, being an important explanation for their biogeography, but is not explored in animals. Our study shows that in the lack of reliable fossil an expanded outgroup calibration can be a better option than relying on geographical calibration. Since the beginning of the work on high-frequency songs of eneopterines as a putative acoustic innovation (Robillard & Desutter-Grandcolas 2004b), the initial circumstances that promoted their origin have remained obscured by the lack of dated phylogenetic and biogeographic context. Elucidating the "when" and the "where" of the origin of this acoustic innovation will help propose new directions for future studies of this multidisciplinary and evolutionary puzzle.

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Figure legends

Figure 1. Phylogenetic relationships and divergence times of Eneopterinae: maximum clade credibility tree with median age and 95% confidence interval estimated with a Bayesian uncorrelated relaxed clock implemented in beast. Circles 'A' to 'E' represent calibration points pre-selected, but only A and B were used in dating analyses. The main taxonomic categories are shown, including the polyphyletic Eneopterini (Eneopterini 1 and Eneopterini 2).

Figure 1. Reconstruction of historical biogeography for Eneopterinae using a stratified dispersal–extinction–cladogenesis (DEC + j) model that accounts for geological history. The top-left box represents the 10 areas implemented in the palaeogeographical model. For each node, a coloured square corresponding to the coloured area in the box represents the inferred area(s) with the highest relative probability in the DEC +J analysis. Present-day distributions of each species are given at the tips by coloured circles corresponding to coloured areas on maps. The time slices of each palaeological period used to measure the dispersal rate are show. A 5-Ma time-scale is placed at the bottom of the chronogram spanning epochs since 90 Ma.

Figure 2. Biogeographical reconstructions for Eneopterinae, with specific palaeogeographical maps. For areas, colour coding is the same as that in Fig. 2. Arrows shows the route of dispersal. In late Cretaceous, two lineages diverged from an ancestor, one diverged in Australia whereas the second colonized the Western hemisphere through South America. In the Paleocene ancestral coming from South East Asia colonize India. In Eocene we have the second colonization of South America of Eneopterinae coming from the boreotropics. During Oligocene we have the colonization of New Caledonia e newly formed islands of Southwest Pacific and South East Asia.

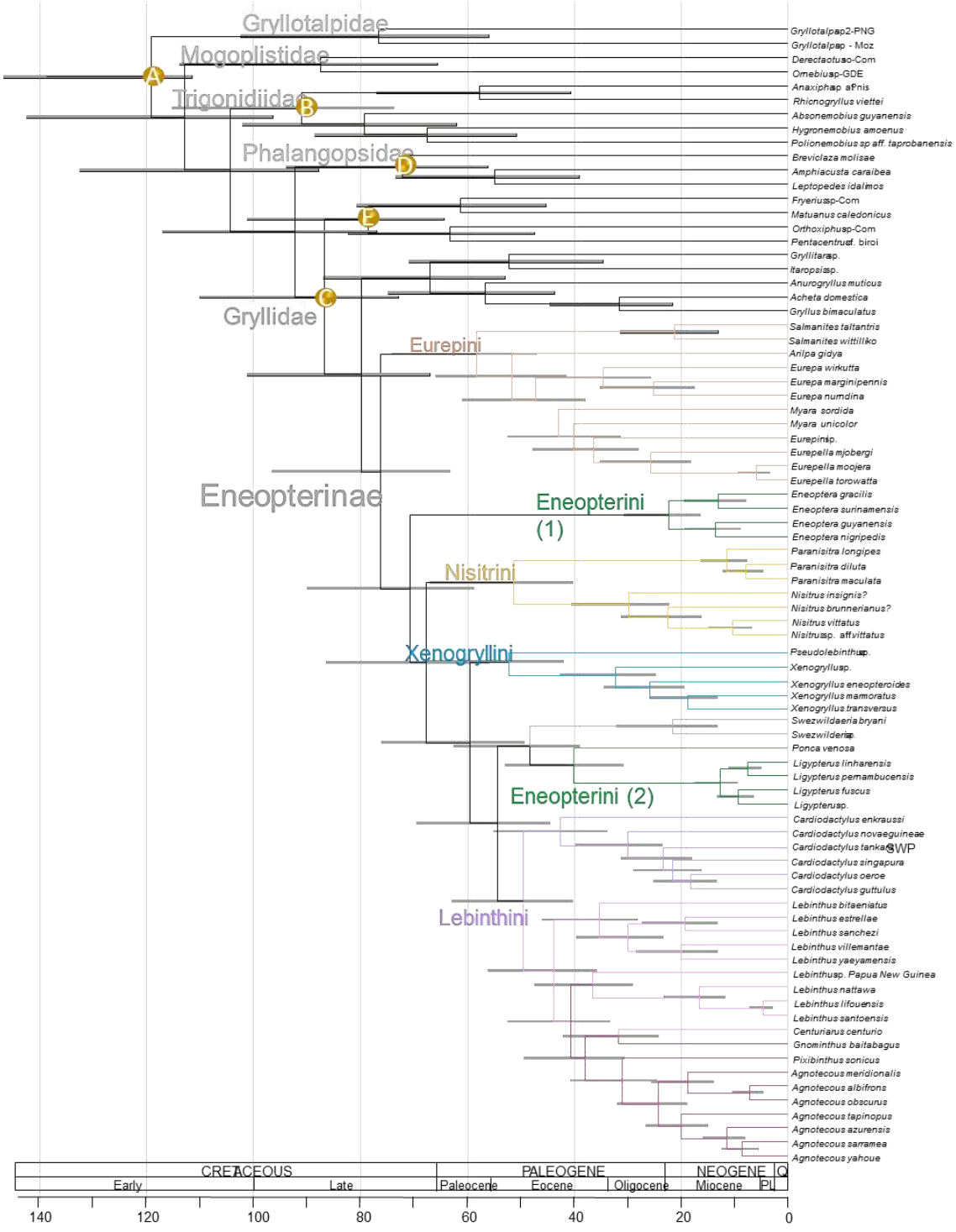
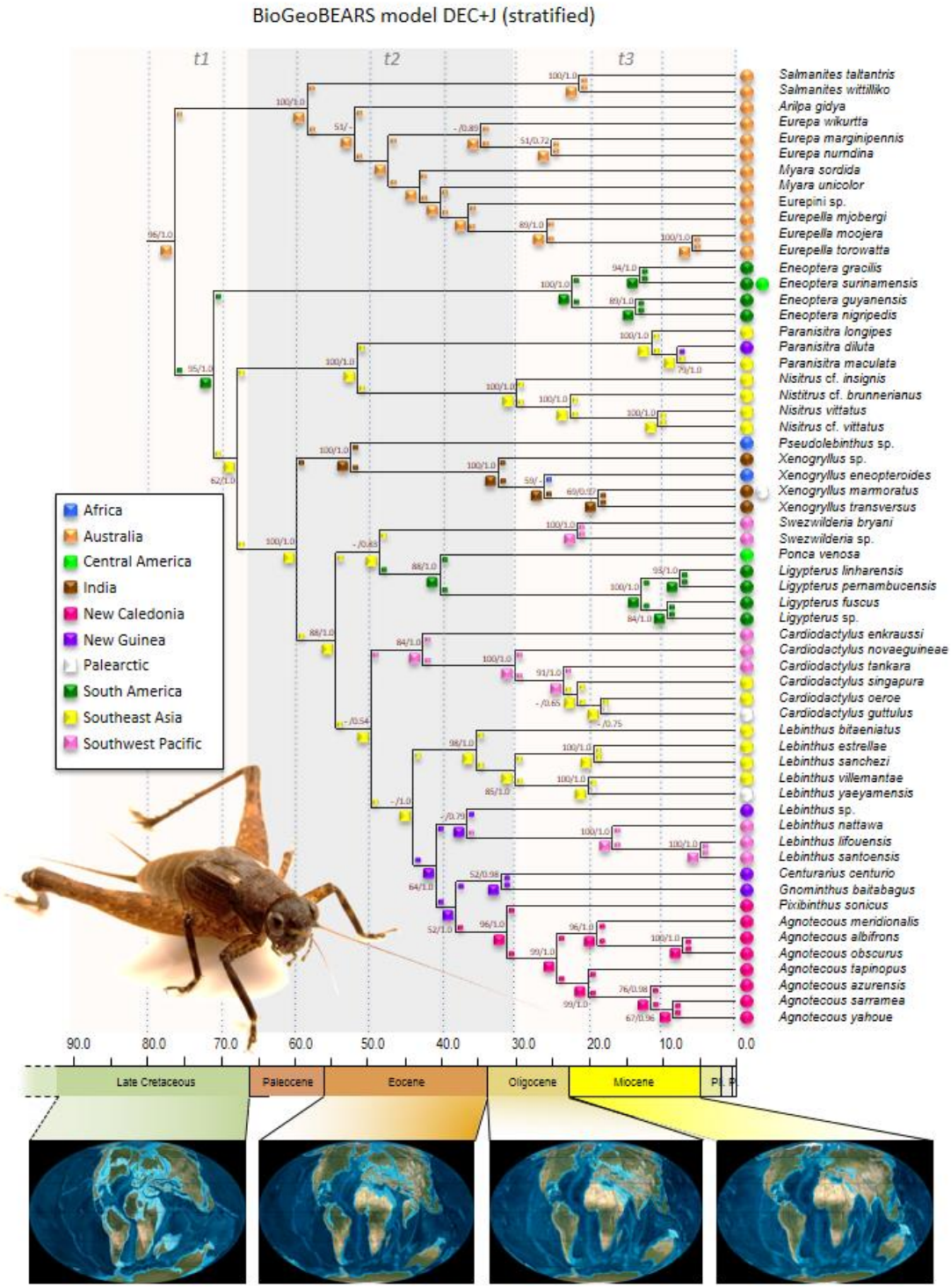

Gryllotalpidae
Mogoplistidae
Trigonidiidae
Phalangopsidae
Gryllidae
Eneopterinae
Eurepini
Eneopterini (1)
Nisitrini
Xenogryllini
Eneopterini (2)
Lebinthini
A
B
C
D
E
Gryllotalpa sp2-PNG
Gryllotalpa sp - Moz
Derectaotus sp-Com
Ornebius sp-GDE
Anaxipha sp aff nis
Rhicnogryllus viettei
Absonemobius guyanensis
Hygronemobius amoenus
Polionemobius sp aff. taprobanensis
Breviclaza molisae
Amphiacusta caraibea
Leptopedes idalmos
Fryerius sp-Com
Matuanus caledonicus
Orthoxiphus sp-Com
Pentacentrus cf. biroi
Gryllita sp.
Itaropsis sp.
Anurogryllus muticus
Acheta domestica
Gryllus bimaculatus
Salmanites taltantris
Salmanites wittilliko
Arilpa gidya
Eurepa wirkutta
Eurepa marginipennis
Eurepa numdina
Myara sordida
Myara unicolor
Eurepini sp.
Eurepella mjobergi
Eurepella mocjera
Eurepella torowatta
Eneoptera gracilis
Eneoptera surinamensis
Eneoptera guyanensis
Eneoptera nigripedis
Paranisitra longipes
Paranisitra diluta
Paranisitra maculata
Nisitrus insignis?
Nisitrus brunnerianus?
Nisitrus vittatus
Nisitrus sp. aff vittatus
Pseudolebinthus sp.
Xenogryllus sp.
Xenogryllus eneopteroides
Xenogryllus marmoratus
Xenogryllus transversus
Swezwildaeria bryani
Swezwilderia sp.
Ponca venosa
Ligypterus linharensis
Ligypterus pernambucensis
Ligypterus fuscus
Ligypterus sp.
Cardiodactylus enkraussi
Cardiodactylus novaeguineae
Cardiodactylus tanka SWP
Cardiodactylus singapura
Cardiodactylus oeroe
Cardiodactylus guttulus
Lebinthus bitaeniatus
Lebinthus estrellae
Lebinthus sanchezi
Lebinthus villemantae
Lebinthus yaeyamensis
Lebinthus sp. Papua New Guinea
Lebinthus nattawa
Lebinthus lifouensis
Lebinthus santoensis
Centuriarus centurio
Gnominthus baitabagus
Pixibinthus sonicus
Agnotecous meridionalis
Agnotecous albifrons
Agnotecous obscurus
Agnotecous tapinopus
Agnotecous azurensis
Agnotecous sarramea
Agnotecous yahoue
CRETACEOUS
Early
Late
PALEOGENE
Paleocene
Eocene
Oligocene
NEOGENE
Miocene
PL
Q
140
120
100
80
60
40
20
0

Figure 1

Figure 2


BioGeoBEARS model DEC+J (stratified)
t1
t2
t3
Africa
Australia
Central America
India
New Caledonia
New Guinea
Palearctic
South America
Southeast Asia
Southwest Pacific
Salmanites taltantris
Salmanites wittilliko
Arilpa gidya
Eurepa wikurtta
Eurepa marginipennis
Eurepa nurndina
Myara sordida
Myara unicolor
Eurepini sp.
Eurepella mjobergi
Eurepella moojera
Eurepella torowatta
Eneoptera gracilis
Eneoptera surinamensis
Eneoptera guyanensis
Eneoptera nigripedis
Paranisitra longipes
Paranisitra diluta
Paranisitra maculata
Nisitrus cf. insignis
Nistitrus cf. brunnerianus
Nisitrus vittatus
Nisitrus cf. vittatus
Pseudolebinthus sp.
Xenogryllus sp.
Xenogryllus eneopteroides
Xenogryllus marmoratus
Xenogryllus transversus
Swezwilderia bryani
Swezwilderia sp.
Ponca venosa
Ligypterus linharensis
Ligypterus pernambucensis
Ligypterus fuscus
Ligypterus sp.
Cardiodactylus enkraussi
Cardiodactylus novaeguineae
Cardiodactylus tankara
Cardiodactylus singapura
Cardiodactylus oeroe
Cardiodactylus guttulus
Lebinthus bitaeniatus
Lebinthus estrellae
Lebinthus sanchezi
Lebinthus villemantae
Lebinthus yaeyamensis
Lebinthus sp.
Lebinthus nattawa
Lebinthus lifouensis
Lebinthus santoensis
Centurarius centurio
Gnominthus baitabagus
Pixibinthus sonicus
Agnotecous meridionalis
Agnotecous albifrons
Agnotecous obscurus
Agnotecous tapinopus
Agnotecous azurensis
Agnotecous sarramea
Agnotecous yahoue
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Late Cretaceous
Paleocene
Eocene
Oligocene
Miocene

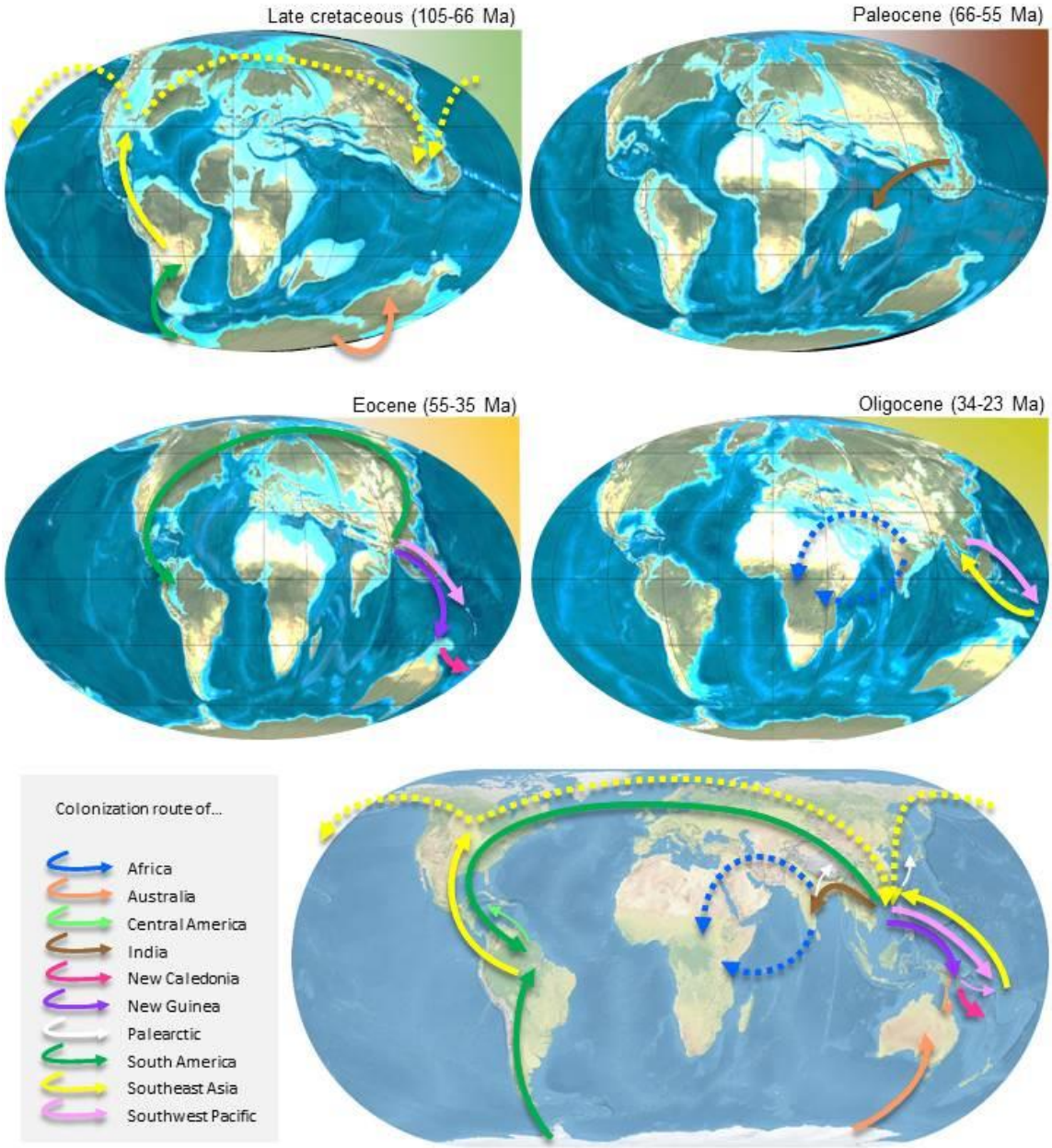

Late cretaceous (105-66 Ma)
Paleocene (66-55 Ma)
Eocene (55-35 Ma)
Oligocene (34-23 Ma)
Colonization route of...
Africa
Australia
Central America
India
New Caledonia
New Guinea
Palearctic
South America
Southeast Asia
Southwest Pacific

Figure 3

Table legends

Table 1. Details about the pre-selected fossil for the outgroup calibration approach performed in this study.

Table 2. Model selection: Bayes factor scores (2 ln B_F) from comparisons of alternative calibration procedures using stepping-stone sampling analyses. The best-fit calibration procedure (according to the criterion 2 ln B_F > 10; Kass & Raftery 1995) is highlighted using bold fonts. The following abbreviations were used: uncorrelated lognormal relaxed clocks (UCLN); random local clocks (RLC); exponential distributions (EXP); uniform distributions (UNI); two fossil constraints (2C); four fossil constraints (4C); not significant (NS).

Species	Age (Ma)	Phylogenetic position	Comment	Locality and stratigraphy	Reference
Archaeogryllotalpoides ornatus Martins-Neto, 1991	122.46 to 112.6	stem Gryllotalpidae	One of the oldest fossils for this group, but the description is based in partial preservation of the tegmens, causing doubts in the identification.	Santana formation, Araripe basin (Crato formation). Ceará, Brazil.	Martins-Neto, 1991
Palaeoscapteriscops cretacea Martins-Neto, 1991	122.46 to 112.6	stem Gryllotalpidae	Fossil from the same locality and age as the previous one. Differs from the preservation of the anterior due to the leg showing two dactylar processes typical in Gryllotalpidae.	Santana formation, Araripe basin (Crato formation). Ceará, Brazil.	Martins-Neto, 1991
Marchandia magnifica Perrichot, Neraudeau, Azar, Menier & Nel, 2002	105.3 to 99.7	stem Gryllotalpidae	We can definitely place this fossil in Gryllotalpidae according to characteristics of the prothoracic leg (tibia with two movable dactylar processes). But *Palaeoscapteriscops cretacea* is older.	Albian part of the Archingeay-Les Nouillers quarry, France.	Perrichot *et al.,* 2002
Cyrtoxipha electrina Gorochov, 2010	20.43 to 13.65	stem Trigonidiidae	The sword shape of the ovipositor of this fossil preserved in amber, enables its placement in the Trigonidiidae stem.	Dominican amber, Dominican republic.	Gorochov, 2010
Cyrtoxipha illegibilis Gorochov, 2010	20.43 to 13.65	stem Trigonidiidae	Description based on a male without the upper body surface and small remnants of the lateral fields of tegmina. The lack of characters causes doubts in the classification of this fossil as a Trigoniididae.	Dominican amber, Dominican republic.	Gorochov, 2010

Baltonemobius fossilis Gorochov, 2010	48.6 to 40.4	stem Trigonidiidae	Maxillary palpi with their terminal segment moderately expanding toward apex, hind tibia with three pairs of spines and with five spurs, hind basitarsus without denticles, with pair of apical spurs.	Dominican amber, Dominican republic.	Gorochov, 2010
Micromacula gracilis Whalley, 1985	196.5 to 189.6	stem Gryllidae	Despite being the oldest "cricket" fossil, the description is based on forewings where no details of the venation can be seen. We could not classify unambiguously this fossil in the Gryllidae group.	Dorset lias, United Kingdom.	Whalley, 1985
Araripegryllus orientalis Gorochov, Jarzembowski & Coram, 2006	136.4 to 130.0	stem Gryllidae	The fossil was described based on a tegmen poorly preserved apically. Its inclusion in the Gryllidae stem is thus uncertain.	Clockhouse Brickworks, United Kingdom.	Gorochov *et al.,* 2006
Liaonemobius tanae Ren, 1998	130.0 to 125.45	stem Gryllidae	The description it's based on an apterous specimen and no other character could conclusively place this fossil in the stem of Gryllidae. The specimen could be a nymph of another family.	Yixian Formation of West Liaoning, China	Ren, 1998
Araripegryllus camposae Martins-Neto, 1987	122.46 to 112.6	stem Gryllidae	The fossil has the characteristic features of a Gryllidae: globular head, prominent eyes, posterior tibia with at least 3 apical spurs.	Santana formation, Araripe basin (Crato formation). Ceará, Brazil.	Martins-Neto, 1987
Eotrella mira Gorochov, 2012	50.3 to 46.2	stem Phalangopsidae	The characters of the fossil are not diagnostic enough to place it in the Phalangopsidae group.	Green River Formation, Wyoming, USA.	Gorochov 2012
Eozacla problematica Gorochov, 2012	*50.3 to 46.2*	stem Phalangopsidae	The description is based on incomplete imprint of body with indistinct venation, without hind part of wings, hind legs and abdomen.	Green River Formation, Wyoming, USA.	Gorochov 2012

Araneagryllus dylani Heads, 2010	20.43 to 13.65	stem Phalangopsidae	The spider-like shape of the body associated with tibia colour pattern and presence of troglobiomorphic characters places this fossil in the Phalangopsidae stem.	Dominican amber, Dominican republic.	Heads, 2010
Proanaxipha madgesuttonae Heads & Penney, 2012	20.43 to 13.65	Stem Pentacentrinae	Head capsule compressed dorsoventrally; lateral field with veins running parallel to the costal margin; presence of small spines above metatibial spurs.	Dominican amber, Dominican republic.	Heads & Penney, 2012

Table 1

Calibration procedures	Estimated mean -lnL	vs. UCLN EXP 2C	vs. UCLN EXP 4C	vs. UCLN UNI 2C	vs. UCLN UNI 4C	vs. RLC EXP 2C	vs. RLC EXP 4C	vs. RLC UNI 2C	vs. RLC UNI 4C
UCLN EXP 2C	**-66783.39**	-	**12.92**	9.34 (NS)	**11.74**	**62.36**	**74.22**	**66.64**	**63.24**
UCLN EXP 4C	-66789.85	-12.92	-	-3.58 (NS)	-1.18 (NS)	50.44	61.30	53.72	50.32
UCLN UNI 2C	-66788.06	-9.34 (NS)	3.58 (NS)	-	2.40 (NS)	53.02	64.88	57.30	53.90
UCLN UNI 4C	-66789.26	-11.74	1.18 (NS)	-2.40 (NS)	-	50.62	62.48	54.90	51.50
RLC EXP 2C	-66814.57	-62.36	-50.44	-53.02	-50.62	-	11.86	4.28 (NS)	0.88 (NS)
RLC EXP 4C	-66820.50	-74.22	-61.30	-64.88	-62.48	-11.86	-	-7.58 (NS)	-10.98
RLC UNI 2C	-66816.71	-66.64	-53.72	-57.30	-54.90	-4.28 (NS)	7.58 (NS)	-	-3.4 (NS)
RLC UNI 4C	-66815.01	-63.24	-50.32	-53.90	-51.50	--0.88 (NS)	10.98	3.4 (NS)	-

Table 2

Supporting Information

a) Taxa used in this study

Table S1. List of taxa of Eneopterinae and outgroups used in this study with geographical distribution and GenBank accession numbers of each marker.

Voucher	Specie	Geographical Distribution	COI	H3	CytB	18S	16S	28S	12S
AalCT	*Agnotecous albifrons*	New Caledonia							
AazPP	*Agnotecous azurensis*	New Caledonia							
Agi	*Arilpa gidya*	Australia							
AmePB	*Agnotecous meridionalis*	New Caledonia							
AobMa	*Agnotecous obscurus*	New Caledonia							
AsaMA	*Agnotecous sarramea*	New Caledonia							
Ayako2	*Agnotecous yahoue*	New Caledonia							
AtaKo2	*Agnotecous tapinopus*	New Caledonia							
CenV	*Cardiodactylus enkraussi*	South West Pacific							
Cgu	*Cardiodactylus guttulus*	Palearctic							
CnMa	*Centuriarus centurio*	New Guinea							
CnoPe	*Cardiodactylus novaeguineae*	South West Pacific (New guinea, New Caledonia, Australia)							
Csi	*Cardiodactylus singapura*	South East Asia							
Csul1	*Cardiodactylus oeroe*	South East Asia							
CtaT	*Cardiodactylus tankara*	South West Pacific							
Egr	*Eneoptera gracilis*	South America							
Egu	*Eneoptera guyanensis*	South America							
Ema	*Eurepa marginipennis*	Australia							
Eni2	*Eneoptera nigripedis*	South America							
Enu	*Eurepa nurndina*	Australia							
EsuPe	*Eneoptera surinamensis*	South America, Central America							
Eto	*Eurepella torowatta*	Australia							
Eursp	*Eurepini sp.*	Australia							

Emj	*Eurepella mjobergi*	Australia
Emo	*Eurepella moojera*	Australia
Ewi	*Eurepa wikurtta*	Australia
LbiS	*Lebinthus bitaeniatus*	South East Asia
LeNC	*Pixibinthus sonicus*	New Caledonia
LesBa	*Lebitnthus estrella*	South East Asia
Lf2	*Ligypterus linharensis*	South America
Lper	*Ligypterus pernambucensis*	South America
LfuPa	*Ligypterus fuscus*	South America
Lmak3	*Lebinthus sanchezi*	South East Asia
Lman	*Ligypterus sp.*	South America
Lli	*Lebinthus lifouensis*	South West Pacific
Lna	*Lebinthus nattawa*	South West Pacific
LpBai	*Gnominthus baitabagus*	New guinea
Lpng1	*Lebinthus sp. Papua New Guinea*	New guinea
LsaV	*Lebinthus santoensis*	South West Pacific
Lvil	*Lebinthus villemantae*	South East Asia
Lyae	*Lebinthus yaeyamensis*	Palearctic
Mso	*Myara sordida*	Australia
Mun	*Myara unicolor*	Australia
Nhy	*Nisitrus insignis?*	South East Asia
Nka	*Nisitrus brunnerianus?*	South East Asia
NviS	*Nisitrus vittatus*	South East Asia
NviSA	*Nisitrus sp. aff.vittatus*	South East Asia
Pdi	*Paranisitra diluta*	New guinea
Plo2	*Paranisitra longipes*	South East Asia
Pma	*Paranisitra maculata*	South East Asia
Pve	*Ponca venosa*	Central America
Sbr	*Swezwildaeria bryani*	South West Pacific
Ssp	*Swezwilderia sp.*	South West Pacific

Sta	*Salmanites taltantris*	Australia
Swi	*Salmanites wittilliko*	Australia
X17	*Pseudolebinthus sp.*	Africa
XenAC	*Xenogryllus eneopteroides*	Africa
XmaCh1	*Xenogryllus marmoratus*	Palearctic, India
Xsp	*Xenogryllus sp.*	India
XtrIn	*Xenogryllus transversus*	India
LDG 047	*Absonemobius guyanensis*	South America
LDG 254	*Gryllotalpa sp2*	New Guinea
LDG 171	*Gryllotalpa sp*	Africa
LDG 097	*Derectaotus SP*	Africa
LDG 184	*Ornebius SP*	Central America
LDG 048	*Hygronemobius amoenus*	South America
LDG 057	*Polionemobius sp aff taprobanensis*	India
LDG 020	*Amphiacusta caraibea*	Central America
LDG 010	*Brevizacla molisae*	South West pacific
LDG 011	*Leptopedetes idalimos*	Central America
LDG 096	*Fryerius sp*	Africa
LDG 220	*Orthoxiphus sp*	Africa
LDG 159	*Pentacentrus cf. biroi*	South West Pacific
LDG 101	*Anaxipha sp affinis nitida*	South America

b) Primers used in this Study

Table S2. Forward (F) and reverse (R) PCR primers used to amplify portions of the mitochondrial (16S, 12S, cytochrome b, cytochrome oxidase) and nuclear (18S, 28S, H3) genes, with their annealing temperature.

Gene	Primer	Sequencia	Referência	Temperatura de Annealing (°C)
12 S	12SF	TACTATGTTACGACTTAT	Kambhampat, 1995	48
	12SR	AAACTAGGATTAGATACCC	Kambhampat, 1995	
16S	16SAG	CGCCTGTTTATCAAAAACATGT	Robillard & Desutter-Grandcolas, 2006	55
	16SBG	AGATCACGTAAGAATTTAATGGTC	Robillard & Desutter-Grandcolas, 2006	
Cyt b	427F	YTWGTWCAATGARTMTGAGG	Robillard & Desutter-Grandcolas, 2006	48
	800R	CCYARTTTATTAGGAATTGATCG	Robillard & Desutter-Grandcolas, 2006	
COI	L2	GCAACGATGATTATTTTCCACT	Nattier *et al.,* 2012	49
	H2	CCTGGTAAAATTAGAATGTAAACTTCTG	Nattier *et al.,* 2012	
18S	A2	ATGGTTGCAAAGCTGAAAC	Smith *et al.,* 2007	52
	9R	GATCCTTCCGCAGGTTCACCTA	Grandcolas *et al.,* 2008	
28S	Rd32b	CCYTGAACGGTTTCACGTACT	Esse estudo	50
	Rd12a	CCCSSGTAATTTAAGCATATTA	Esse estudo	
H3	HexAF	ATGGCTCGTACCAAGCAGACGGC	Murienne *et al.,* 2009	58
	HexAR	ATATCCTTGGGCATGATGGTGAC	Murienne *et al.,* 2009	

c) Paleogeographical model

About time slice I (Late Cretaceous 80-65 Ma):

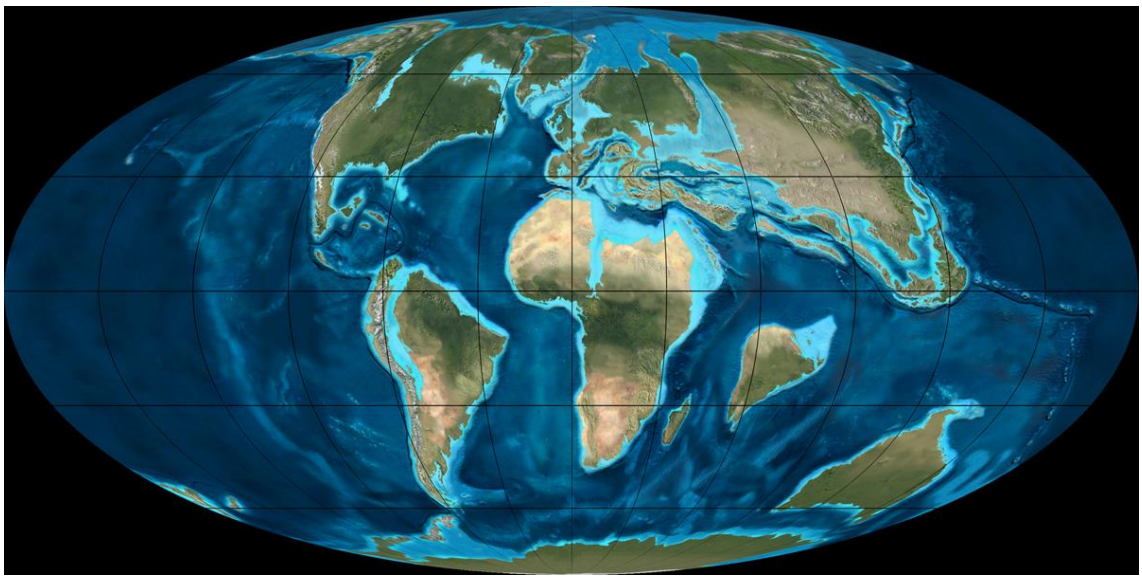


Figure S1. Paleogeographic map reconstructed from Blakey (2008) representing late cretaceous (80-65 Ma)

Dispersal between Gondwanan landmasses was still allowed because of the connection South America- Australia-New Guinea by the Drake Passage (probably lasted until 60 Ma) and by the Kerguelen Plateau that still connected India-Australia (Sanmartín & Ronquist, 2004). A dispersal movement in SWP, CA and NC is disallowed because at this time these islands were not formed (Neall & Trewick, 2008).

Table S3. Dispersal rate in Late Cretaceous.

	AFR	IND	AUS	NC	NG	NSA	CA	SEA	SWP	EP
AFR	-	0,01	0,01	0	0,01	0,01	0	0,01	0	0,01
IND		-	1	0	1	0,01	0	0,01	0	0,01
AUS			-	0	1	0,01	0	0,01	0	0,01
NC				-	0	0	0	0	0	0
NG					-	0,01	0	0,01	0	0,01
NSA						-	0	0,01	0	0,01
CA							-	0	0	0
SEA								-	0	1
SWP									-	0
EP										-

About time slice II (Paleocene/Eocene 60-30 Ma):

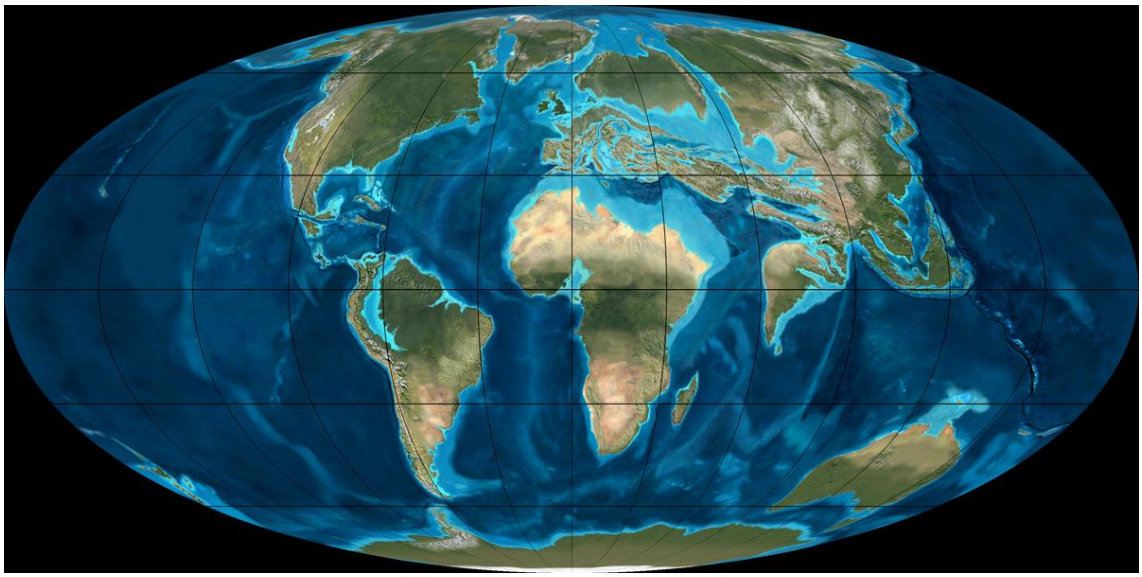


Figure S2. Paleogeographic map reconstructed from Blakey (2008) representing Paleocene/Eocene (60-30 Ma)

In this time slice, India and Africa arrived in contact with Holarctic and dispersal between these three continents is allowed (Sanmartín & Ronquist, 2004). South-America-Antartica-Australia-New Guinea were still considered as connected by the Antarctic Peninsula (Sanmartín & Ronquist, 2004). Australia and New Guinea were still connected to South America through Antarctica. The former two began to separate from Antarctica around 50 Ma (Sanmartín & Ronquist, 2004). New Caledonia emerged from the ocean during the late Eocene. In this time slice NC probably began to be recolonized by the surround areas (SWP, NG, and Australia most likely). Some South Pacific islands were formed in this period, allowing dispersal with the neighbouring areas.

Table S4. Dispersal rate in Paleocene/Eocene.

	AFR	IND	AUS	NC	NG	NSA	CA	SEA	SWP	EP
AFR	-	0,01	0,01	0,01	0,01	0,01	0	0,01	0,01	1
IND		-	0,01	0,01	0,01	0,01	0	1	0,3	1
AUS			-	0,01	1	0,01	0	0,01	0,01	0,01
NC				-	0,01	0,01	0	0,01	0,01	0,01
NG					-	0,01	0	0,01	0,01	0,01
NSA						-	0	0,01	0,01	0,01
CA							-	0	0	0
SEA								-	1	1
SWP									-	1
EP										-

About time slice III (Oligocene/Neogene 30-0 Ma):

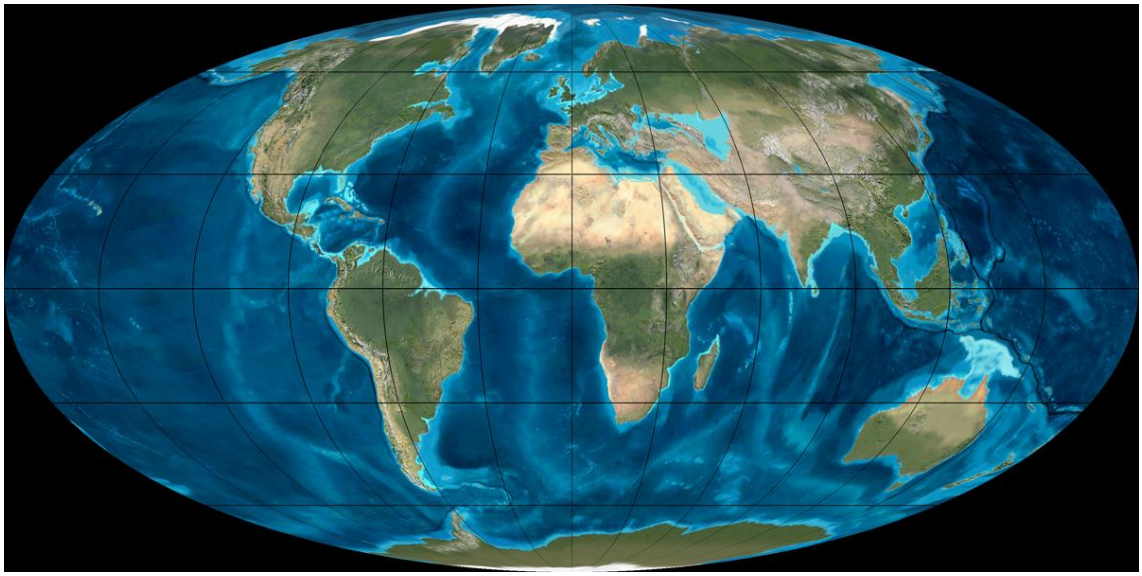


Figure S3. Paleogeographic map reconstructed from Blakey (2008) representing Oligocene/Neogene (30-0 Ma)

In this period, except for IND- AFR-HOL and NSA-CA, all Gondwana landmasses were separated. It also began an intensive dispersal between the newly formed islands of SWP, SEA and CA and the neighboring areas.

Table S5. Dispersal rate in Oligocene/Neogene

	AFR	IND	AUS	NC	NG	NSA	CA	SEA	SWP	EP
AFR	-	1	0,01	0,01	0,01	0,01	0,01	0,3	0,01	0,3
IND		-	0,01	0,01	0,01	0,01	0,01	0,3	0,01	1
AUS			-	0,03	0,7	0,01	0,01	0,01	0,01	0,01
NC				-	0,01	0,01	0,01	0,01	0,3	0,01
NG					-	0,01	0,01	0,01	0,3	0,01
NSA						-	1	0,01	0,01	0,01
CA							-	0,01	0,01	0,01
SEA								-	0,3	0,7
SWP									-	0,01
EP										-

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c) Maximum Likelihood

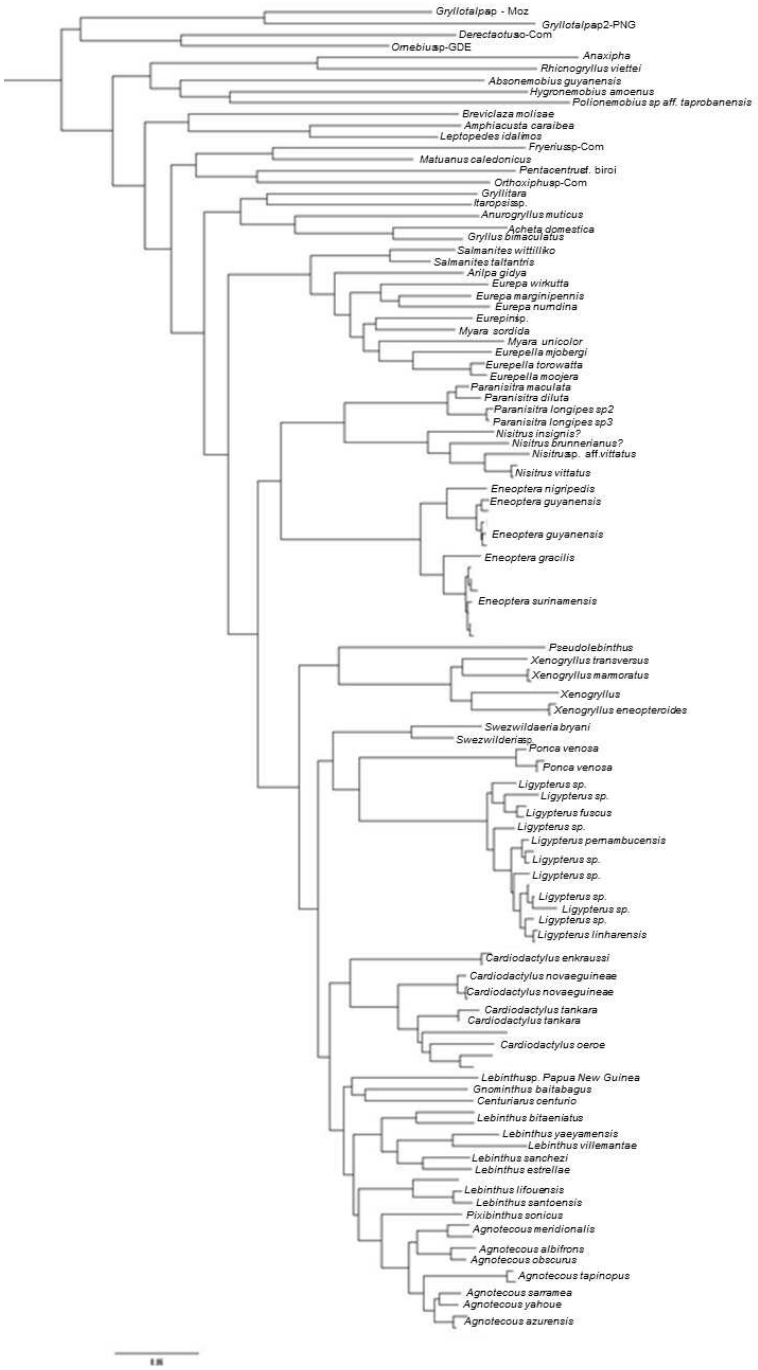

Figure S4. Phylogenetic relationship of Eneopterinae species inferred by Maximum Likelihood based on combined dataset (COI, CytB, 12S, 16S, 12S, 18S, 28S and H3)

d) Bayesian Inference

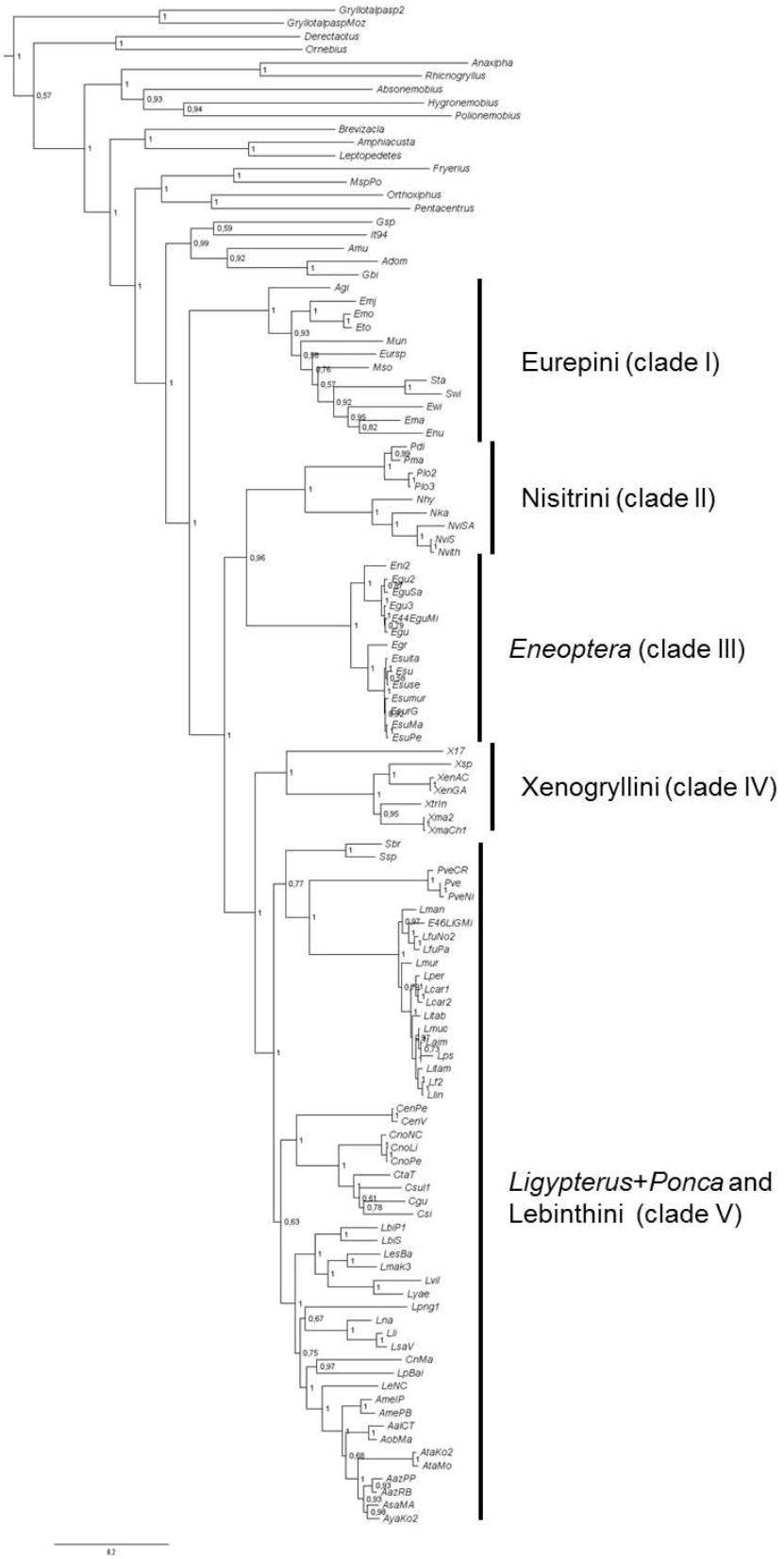

Figure S5. Phylogenetic relationship of Eneopterinae species inferred by Bayesian analyses based on combined dataset (COI, CytB, 12S, 16S, 12S, 18S, 28S and H3). The major taxonomic categories are show.

CHAPTER TWO

Multiple origins of high-frequency communication in extant crickets (Eneopterinae): Did it all happen in the Neotropics?

Manuscript in preparation, to be submitted to *Biological Journal of the Linnean Society*.

Chapter 2: Multiple origins of high-frequency communication in extant crickets (Eneopterinae): Did it all happen in the Neotropics?

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Keywords: cricket, calling song, dominant frequency

Abstract

We studied the bioacoustics of the Neotropical genera of Eneopterinae crickets and discuss the evolution of the single case of high-frequency calling known in crickets. The new bioacoustic data of Neotropical Eneopterinae are replaced in the updated phylogenetic context of the subfamily in order to address the timing and geographical origin of high-frequency signaling in crickets. Did high-frequency communication originate in the Neotropics or in the Pacific Islands as previously hypothesized, once or multiple times?

Introduction

The Eneopterinae crickets (Gryllidae) have recently been extensively studied for their capacity to use high-frequency calling signals to communicate (Robillard & Desutter-Grandcolas 2004b, Robillard *et al.,* 2007, 2013). They are indeed the only known group of crickets presenting such signals, and some species even produce exclusively ultrasounds reaching more than 28 kHz (Robillard *et al.,* 2007; Anso *et al.,* unpublished). This is far above the limits of normal cricket songs, that usually vary between 2 and 8 kHz (e.g. Gerhardt & Huber 2002), and has been hypothesized as a key innovation for an adaptive radiation of Eneopterinae toward high-frequency communication (Robillard & Desutter-Grandcolas 2004b). A recent multidisciplinary study combining phylogeny, neurophysiology and bioacoustics revealed that these high-frequency signals are in fact the first step of a completely new system of communication adopted by Lebinthini species, where females lack phonotaxis but reply to the

male high-frequency song by producing vibrations transmitted to the male throught the plant substrate (ter Hosftede *et al.,* in press).

On the other hand, the species *Eneoptera guyanensis* Chopard (Eneopterini) has long been acknowledged to possess high-frequency components in its peculiar calling song: *E. guyanensis* alternates between high and low calling frequencies complex movements of stridulation (Robillard & Desutter-Grandcolas 2011), and it is likely that the species *E. nigripedis* Robillard does the same, given the modifications of its stridulatory file.

Recently, the calling songs of several eneopterine species from the Neotropics have been reinterpreted in the light of recent findings concerning the mechanism of sound production involved in high-frequency songs in these crickets (Robillard et al. 2015). Here we replace these new information in a phylogenetic context to address the timing and geographical origin of high-frequency signaling. According to the expanded phylogeny of the subfamily presented in Vicente et al. (in prep.), there are strong relationships between the high-frequency Lebinthini and the Neotropical clade *Ponca-Ligypterus*, while the genus *Eneoptera* diverged very early and independently in the evolution of Eneopterinae. These results suggest the placement of *Ponca-Ligypterus* in Lebinthini. We propose here to investigate the timing and geographical origin of high-frequency communication in these crickets, and in particular the possibility that high-frequency communication originated in the Neotropics. Since the beginning of the work on high-frequency songs of eneopterines as a putative acoustic innovation (Robillard & Desutter-Grandcolas 2004b), the initial circumstances that promoted their origin have remained obscured by the lack of dated phylogenetic and biogeographic context. Elucidating the "when" and the "where" of the origin of this acoustic innovation, in addition to the recent

biomechanical and neuroethological advances will help to propose new directions for future studies of this multidisciplinary and evolutionary puzzle.

Materials and methods

We combined the dated phylogeny and the results of the biogeographical analyses from Vicente et al. (in prep.) to the frequency range of each species in the tree in order to analyze the origin of high-frequency signals.

The acoustic information for each species was obtained from the literature, from unpublished data (T. Robillard), and from the analysis of the Neotropical species (Robillard et al. 2015). We considered the acoustic information as one binary character: frequency in the normal range of crickets (<8 kHz: state 0), high-frequency (above 10 kHz: state 1). We consider the species having lost the acoustic communication as missing data since the frequency values of the song (states low or high) are not homologous with absence of calling song. This character is analyzed here with parsimony only; analyses taking into account the continuous frequency values with probabilistic analyses will be developed in further studies.

Results & discussion

The type of song frequency analysed in a parsimony framework, our study gives only one scenario for the origin of high-frequency songs in Eneopterinae, with two independent origins as suggested before (Robillard *et al.,* 2007):

1) One origin of high frequency concerns the particular song of *Eneoptera guyanensis*. According to the results of Vicente *et al*., in prep, the origin of *Eneoptera* species is quite recent and would have occurred in South America

during early Miocene (18-23 Ma). As presented above, the song of *E. guyanensis* alternates high frequency and low frequency due to a characteristic stridulatory file structure: one section with teeth widely separated and the other with teeth close together (Desutter-Grandcolas, 1998). The high frequency part has a dominant frequency of almost 30 kHz. Despite the complexity of the sound production system, this case of high frequency has little impact in terms of acoustic diversification, since it concerns only one recent species, or possibly two, given that the sister species of *E. guyanensis*, *E. nigripedis,* found in Peru and south-west of Brazil, also possesses modified stridulatory structures as in *E. guyanensis* (Robillard & Desutter-Grandcolas 2005). Studies strongly suggest that the other species of *Eneoptera* also possess calling song with similar features as *E. guyanensis* (Robillard *et al.,* 2015). Particular attention should then be directed to these species aiming to better understand the evolution of high-frequency calls.

2) The second occurrence of high-frequency signals in Eneopterinae is far more important in terms of phylogenetic diversification. Indeed, it concerns a large clade corresponding to ((*Swezwilderia* (*Ponca-Ligypterus*)) Lebinthini) (ie. in the Lebinthini *sensu lato).* Unlike *Eneoptera* which comprises only a few species despite its early divergence, Lebinthini is the most diverse group in Eneopterinae and comprises currently ca. 150 species which are diversified morphologically and distributed in a vast region encompassing Southeast Asia, New Guinea, South West Pacific, Northern Australia, New Caledonia, and, according to our results, Northern South-America and Central America if we consider the genera *Ponca* and *Ligypterus* as Lebinthini.

All genera and the studied species from this clade, i.e. *Cardiodactylus Lebinthus Agnotecous, Gnominthus, Pixibinthus,* use only high-frequency communication, sometimes in ultrasonic levels (Robillard 2009; Anso *et al.,*

submit). Together with colonization of areas that may provide access to resources that were unavailable before, such as new islands, high-frequency calls appears to have played an important role in the diversification of Lebinthini. In this context, Robillard & Desutter-Grandcolas (2004a) proposed that this lineage experienced an adaptive radiation as a consequence of the evolution of high-frequency songs. Our phylogenetic hypothesis, recovering *Ligypterus-Ponca* as sister clade, together with bioacoustics results from Robillard *et al.,* (2015), strongly indicate the close relationship between these two groups and displaces the origin of the adaptive radiation from the Pacifc region to the common ancestral area of the Neoptropical clade and the rest of the Lebinthini.

According to Vicente et al. (in prep.), this common origin high-frequencies as only dominant frequency of the song is much older than the case of *Eneoptera*, and would have occurred in Southeast Asia between late Paleocene and early Eocene (54-60 Ma). The Southeast Asia area involved in this shift from low to high frequency is however likely to represent a larger past distribution of the eneopterines in the Boreotropical region, possibly encompassing the whole Holarctic region, since the Lebinthini recolonized South-America during the Eocene, in parallel with multiple colonization events of the South West Pacific and New Guinea.

Strikingly, this date coincides with the development of high frequencies in tettigoniids, which have developed a high faunal diversity upon the use of high-frequency sound communication. This coincidence in time of origin of high-frequencies suggests that tettigoniids and eneopterine crickets may have independently developed a similar solution while confronted to a similar problem, such as predation by bats, which diversification is also often dated back to ca. 60 Ma (Gu *et al.,* 2012).

Conclusion

In this paper we clarify the evolution of high frequency songs in Eneopterinae crickets, inferring the dating and origin centers of this characteristic. The access of new resources and the success in this new ability of communication (influencing the scape from predators and parasites, increase calling effectiveness and pre-zigotic isolation) seems to have played an important role in diversification of the most speciose group in Eneopterinae, the Lebinthini. Given the evolutionary and ecological importance showed here and in other studies (Robillar & Desutter-Grandcolas, 2004b, Robillard *et al.,* 2007, Robillard *et al.,* 2013) we believe that high frequency song needs to be better explored in other crickets group.

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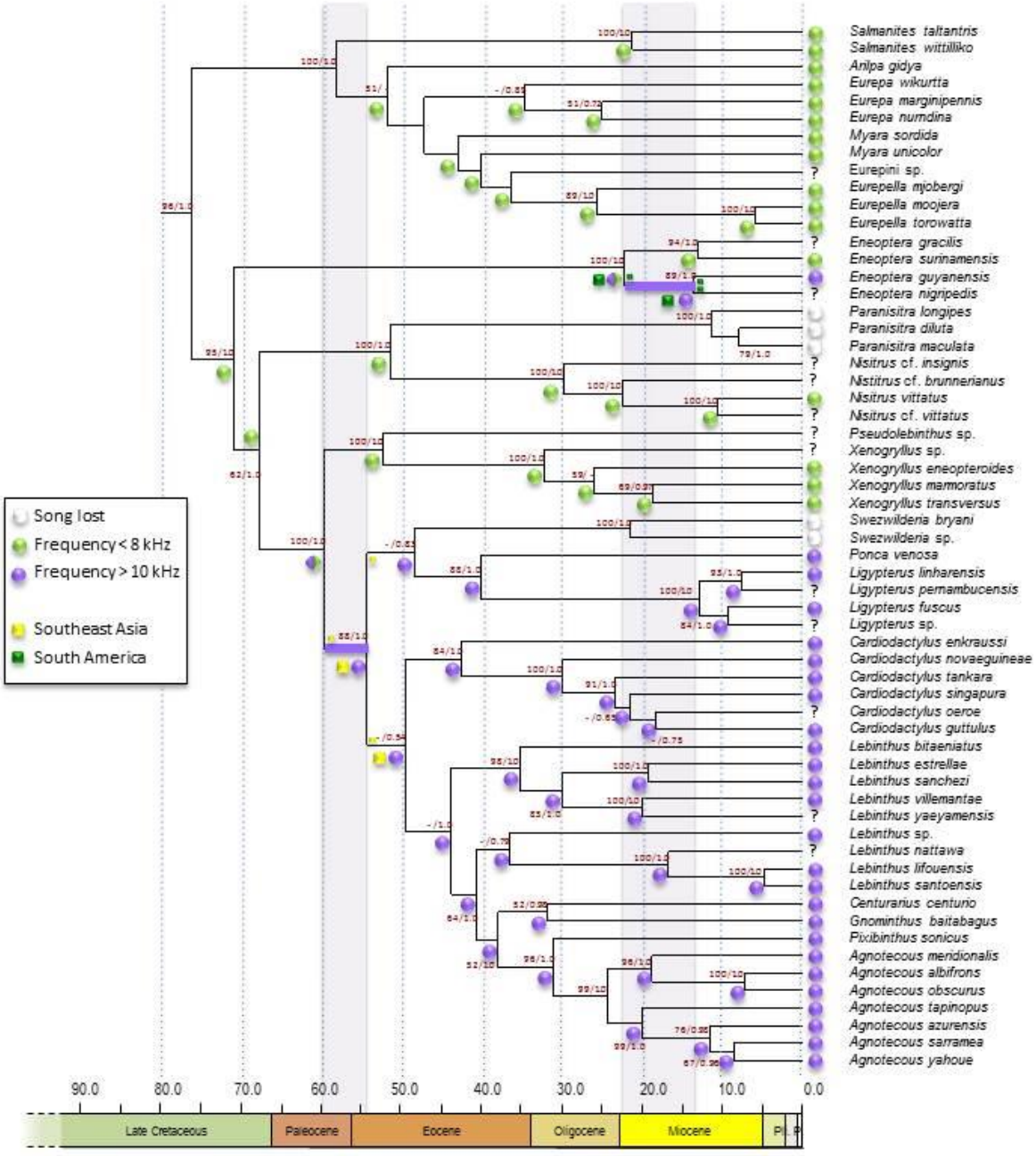
Figure legend

Figure 1. Distribution of frequency range in reference to the phylogeny of Eneopterinae crickets (modified after Vicente *et al.,* in prep.). Interrogation marks are not recorded species.

Figure 1


Song lost
Frequency < 8 kHz
Frequency > 10 kHz
Southeast Asia
South America
Salmanites taltantris
Salmanites wittilliko
Anlpa gidya
Eurepa wikurtta
Eurepa marginipennis
Eurepa numdina
Myara sordida
Myara unicolor
Eurepini sp.
Eurepella mjobergi
Eurepella moojera
Eurepella torowatta
Eneoptera gracilis
Eneoptera surinamensis
Eneoptera guyanensis
Eneoptera nigripedis
Paranisitra longipes
Paranisitra diluta
Paranisitra maculata
Nisitrus cf. insignis
Nistitrus cf. brunnerianus
Nisitrus vittatus
Nisitrus cf. vittatus
Pseudolebinthus sp.
Xenogryllus sp.
Xenogryllus eneopteroides
Xenogryllus marmoratus
Xenogryllus transversus
Swezwilderia bryani
Swezwilderia sp.
Ponca venosa
Ligypterus linharensis
Ligypterus pernambucensis
Ligypterus fuscus
Ligypterus sp.
Cardiodactylus enkraussi
Cardiodactylus novaeguineae
Cardiodactylus tankara
Cardiodactylus singapura
Cardiodactylus oeroe
Cardiodactylus guttulus
Lebinthus bitaeniatus
Lebinthus estrellae
Lebinthus sanchezi
Lebinthus villemantae
Lebinthus yaeyamensis
Lebinthus sp.
Lebinthus nattawa
Lebinthus lifouensis
Lebinthus santoensis
Centurarius centurio
Gnominthus baitabagus
Pixibinthus sonicus
Agnotecous meridionalis
Agnotecous albifrons
Agnotecous obscurus
Agnotecous tapinopus
Agnotecous azurensis
Agnotecous sarramea
Agnotecous yahoue
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Late Cretaceous
Paleocene
Eocene
Oligocene
Miocene

CHAPTER THREE

Exploring the diversity in the cryptic species genus Ligypterus

(Eneopterinae: Orthoptera)

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Exploring the diversity in the cryptic species genus *Ligypterus* (Eneopterinae: Orthoptera)

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Abstract

The use of species delimitation methods based on different kinds of data are promising tools to assess species boundaries in taxa with confusing taxonomy. Here, we explored the species diversity in *Ligypterus,* a Neotropical cricket genus containing a series of possibly cryptic species showing a wide array of morphological variation. Using specimens from eight localities across Brazil and French Guiana, we used two molecular markers, the mitochondrial gene CytB and the nuclear gene 28S to delimitate species under different DNA based discovery methods (Automatic Barcode Gap (ABGD), General Mixed Yule-Coalescent (GMYC) and Poisson Tree Process (PTP)) and Geometric morphometrics (GM). The results were very congruent, giving from 10 to 14 putative species, which were then tested with a geometric morphometric approach. Despite the limited sampling, GM showed consistent results with the DNA-based approaches. Considering all the analyses in a conservative way, our results estimate that the sampled localities present between 5 to 10 putative species of *Ligypterus* crickets. Given that *Ligypterus* has currently 5 species described, our estimates indicate that the real diversity of the genus may be up to twice that number according to our sampling.

Introduction

Species is the basic unit of biological diversity and is directly involved in ecological and evolutionary processes. Given its importance, it is fundamental to have a solid concept for such a unit. However, researchers cannot reach a consensus when it comes to define and delimit species (de Queiroz 2007, Mayden 1997). Estimates of biodiversity on the Planet (Mora *et al.,* 2011) frustrate any researcher when confronted with the risk of extinction and the slowness of traditional taxonomy to deal fully with this unknown diversity (Wheeler 2004).

The challenge gets bigger when we include in this unknown diversity cryptic species that are morphologically indistinguishable or highly similar (Bickford *et al.,* 2006; Pfenninger & Schwenk, 2007). Mayr (1997) proposed that cryptic species are *in status nascendi*, *i.e.* diverged recently and had insufficient time to differentiate. On the other hand, Bickford *et al.,* (2006) proposed that cryptic species could be due to a morphological *stasis,* caused by the stabilizing selection of morphological traits due to extreme environmental conditions. On the other hand, many studies point out more complicated evolutionary stories involving dynamic geographical barriers and gene flux to interpret cases of cryptic species. Cryptic species are a common phenomenon representing a substantial portion of taxa in all geographic regions (Pfenninger & Schwenk, 2007). Given their deep implication in ecological and evolutionary processes, it is necessary to estimate the cryptic diversity to better understand ecosystem functioning. Ultimately, complexes of cryptic species can provide priceless

information about the speciation and faunal diversification processes in time and space.

These difficulties encouraged the integration of taxonomy with approaches using different kinds of data, including DNA sequences from both nuclear and mitochondrial origin in addition to traditional morphological and anatomical characters to test species boundaries and identification (Dayrat 2005).

The group under study in this article belongs to the Neotropical cricket genus *Ligypterus* that belongs to the subfamily Eneopterinae and encompasses five described species based mainly on characters of the male genitalia (Robillard & Desutter-Grandcolas 2005). The genus is distributed widely in Brazil and French Guiana, although few localities have been documented so far. The remarkable similarity between the species of *Ligypterus* and, at the same time, subtle morphological variability within species along their distribution makes species circumscription and identification a hard task.

Recently, *Ligypterus* has been the target of studies regarding the evolution of high-frequency songs (Robillard *et al.,* 2015). According to Vicente *et al.* (in prep.), the genus belongs to Lebinthini, a widely distributed tribe with interesting migration histories. To better analyze these findings and their evolutionary and ecological implications in particular for future studies at the level of the Neotropical fauna, it is important to clarify the current diversity.

In this study, we explored the diversity in *Ligypterus* using different species discovery approaches in an integrative way. We analyzed samples from eight localities from the eastern coast of Brazil and from the Amazon Forest (Manaus and French Guiana). After a critical examination of the traditional morpho-anatomical characters used for cricket taxonomy, we used two molecular markers, the mitochondrial gene CytB and the nuclear gene 28S under different

DNA based species discovery methods (Automatic Barcode Gap, General Mixed Yule-Coalescent and Poisson Tree Process: see methods for details). The resulting hypotheses of species boundaries were then tested with a geometric morphometric approach and discussed according to the geographical context.

Material and methods

Samples

We surveyed specimens of *Ligypterus* from eight localities in Brazil and French Guyana (Figure 1). We gathered tissue samples from 74 individuals for DNA sequencing, considering the most recent specimens available independently of sex. For geometric morphometric analysis, we extracted the right wing of 74 specimens, but exclusively from male specimens, for sake of comparison. For this reason, although the same number or specimens were studied, different individuals were used in both methods. This material was obtained from the Orthoptera's collection of UNESP, Botucatu, São Paulo, or collected during field expeditions of the Brazilian Biota of Orthoptera (Research team, http://www.orthoptera.com.br/pt-BR/). We also included in our analyses sequences of previous studies with Eneopterinae (*Ligypterus fuscus*, *Ligyperus pernambucensis* and *Ligypterus linharensis)* from the collections of the Muséum national d'Histoire naturelle, Paris (MNHN).

Molecular Phylogenetic Reconstruction

The DNA extraction of specimens followed the extraction protocol of Waldschmidt *et al.,* (1997), with slight modifications. For collection samples, DNA extraction was performed using the QIAamp DNA MICROKIT (QIAGEN) following the manufacturer's instructions. We analyzed 400 base pairs (bp) of the 28S ribosomal RNA and ~350bp cytochrome b (CytB). In addition to the

sequences generated by us for this study, we obtained sequences available on GenBank from previous studies in Eneopterinae (*L. fuscus*, *L. pernambusensis* and *L. linharensis*). As outgroup we choose two other species of Neotropical eneopterines from two different genera, one distantly related to *Ligypterus*, *Eneoptera surinamensis*, and *Ponca venosa*, from the sister genus of *Ligypterus.*

The amplification chain reaction (PCR) consists of an initial denaturation step at 94 °C for 3 min for separation of the DNA strands. Subsequently, 30 to 40 cycles of amplification (denaturing at 94 °C for 30s, annealing at 48-58 °C for 40s) and a final step at 72 °C for 5 minutes. Some samples of *CytB* had unspecific bands. We purified the DNA fragment from the agarose gel using the PureLink ® Quick Gel Extraction Kit. The fragments were sent to sequencing in Macrogen (Korea) and Eurofins (France).

We used the software MUSCLE (Edgar, 2004), implemented in MEGA 6.0 (Tamura *et al.,* 2013) to align the sequences. With MEGA we also checked if cytochrome b sequences were congruent with codon reading frame.

We used the software Partition Finder v 1.1.1 (Lanfear *et al.,* 2012) with the Bayesian Information Criterion (BIC) (Schwarz, 1978) to infer the best partitioning scheme and substitution model for MrBayes and BEAST. For that, CytB was separated according to codon position. We conducted a maximum likelihood analysis in the webserver of IQtree (http://www.cibiv.at/software/iqtree) (Nguyen *et al.,* 2015), with automatic substitution model selection and best partitioning scheme selection.

Species discovery Methods

We used different species discovery methods to try to estimate the number of different species among the sampled localities. Species delimitation methods

can be defined according to *a priori* information about input grouping of samples. If samples cannot be assigned to a group *a priori*, the method is described as a species discovery methods, wherease methods where the samples are assigned to a specific groups before the analysis are described as species validation methods (Ence & Carstens 2011). Three discovery methods based on DNA that gained popularity in recent years are Automatic Barcode Gap Discovery (ABGD), General Mixed Yule Coalescent Model (GMYC) and Poisson Tree Process (PTP).

- Automatic Barcode Gap Discovery (ABGD)

ABGD is a method that uses DNA sequences as input dataset and group them into hypothetical species based on the barcode gap (Puillandre *et al.,* 2012). It relies on the assumption that sequences sampled within the same species are always more similar than sequences sampled from different species. Three key parameters have to be estimated before the analysis: (i) *X*, which is an estimate of gap width, (ii) minimum and (iii) maximum values of intraspecific divergence (*P*). After estimating the barcode thresholds, the dataset is partitioned into the maximum number of groups (i.e. putative species) such that the distance between two sequences taken from distinct groups will always be larger than a given threshold distance (i.e. barcode gap). To partitioned the dataset, ABGD employs a two-phase system which (i) initially divides sequences in groups based on statistically inferred barcode gap (i.e., initial partitioning), and (ii) conducts recursive splitting based on the initial threshold to each cluster of the primary partition (i.e., recursive partitioning). The results are displayed in three separate graphs (i) a histogram of distances, showing the barcode gap, (ii) a ranked pairwise differences and (iii) the automatic partition graph reporting the number of groups

inside the partition (initial and recursive) as a function of the prior limit between intra and interspecific divergence.

For our study, we separate the input dataset in $CytB_{nosing}$ (44 sequences in total, without the singletons sequences from *Ligypterus fuscus*, *Ligyperus pernambucensis* and *Ligypterus linharensis* from previous studies) and $CytB_{sing}$ (48 sequences in total, with the singletons sequences from previous studies). The analyses were performed on 07-10 July 2015 on the web interface (http://wwwabi.snv.jussieu.fr/public/abgd/). We ran ABGD with prior intraspecific divergences (P) of 0.005–0.1 (divided into 20 partitions). For each dataset, we tested the impact of width gap in the analysis running the default value (1.5) and 0.75 with Kimura 2-Parameter (K2P) as the distance metric.

- General Mixed Yule-Coalescent (GMYC)

In a phylogenetic tree, the branch lengths between species are determined by speciation and extinction rates (macroevolution), whereas branch lengths within species reflect coalescence processes (microevolution). Pons *et al.,* (2006) developed a statistical framework for estimating the predicted shift in this dynamic of branching associated with species boundaries. GMYC is a coalescent-based method that uses phylogenetic trees as input dataset. It determines the locations of ancestral nodes that define putative species and applies a likelihood ratio test to assess the fit of the branch lengths to a mixed lineage birth-population coalescence model (Pons *et al.,* 2006). The number and composition of putative species is inferred by counting the lineages crossing this transition point. GMYC demands an ultrametric tree as input to run the analyses. The results are separated in number of clusters (groups including more than two samples) and number of entities (number of all delimited species including singletons).

We estimated the ultrametric CytB tree with Beast v1.8.2 (Drummond *et al.,* 2002-2015). Selecting the priors and parameters to construct the tree is a critical step for the method (Ceccarelli *et al.,* 2012, Tang *et al.,* 2014). Previous studies show that different priors to construct the ultrametric tree can lead to different delimitation results in GMYC (Astrin *et al.,* 2011, Tang *et al.,* 2014). Given that, we combined sets of two different clocks (strict or uncorrelated clock lognormal) and three different tree priors (Yule, coalescent, birth/death) in our analyses. As minimum age constraints, we use a secondary calibration from Vicente *et al.,* (in prep.) under an exponential prior. Default values were used for the remaining priors. We ran chains of 20 million generations, sampled every 1000 generation and burning of 2000. Chain mixing and convergence were evaluated in Tracer v1.5 (Rambaut and Drummond, 2003), considering effective sample size (ESS) values > 200 as a good indicator. Maximum clade credibility trees were selected from the combined tree files in TreeAnnotatot v1.8.2 (Rambaut & Drummond 2002-2015). We subjected the ultrametric phylogenies to GMYC analyses with single threshold implemented in the SPLITS package (available from http://r-forge.r-project.org/projects/splits/) with the program R 3.2.1 (R Core Development Team, 2015). We didn't performed a multiple threshold setting once this analysis has shown low accuracy to correctly delimitate species (Tang *et al.,* 2014).

- Poisson Tree Process (PTP)

PTP is also a coalescent-based method that uses a phylogenetic tree as input dataset (Zhang *et al.,* 2013). It differs from GMYC because it uses the number of substitution to identify the transition from species-level to population-level processes (Zhang *et al.,* 2013) rather than branching events. PTP relies on the assumption that the number of substitutions within species is significantly

lower than the number of substitutions between species. PTP has the key advantage of not depending on an ultrametric tree, which is a computationally demanding and error prone process. However, speciation and coalescence are not necessarily related to how many substitutions occur in marker genes (Tang *et al.,* 2014).

For our analyses in PTP, we removed the non-unique haplotypes as suggested by Tang *et al.,* 2014. A RaxML analysis is the most recommended input tree for PTP (Tang *et al.,* 2014), but this software only runs with GTR model. To test how PTP responds to different phylogenetic methods and substitution models, we conducted Bayesian and ML analysis with different datasets and substitution models. The ML analysis was performed in IQtree with 1000 replicates using (i) GTR and (ii) partitioned CytB according to codon position with substitution model selected automatically by IQtree (HKY: CytB_1, K2P+I: CytB_2, F81: CytB_3). For the Bayesian analyses, four Markov chains were run simultaneously for 50 million generations, sampling every 100 generations. The first 25000 trees generated were discarded as burn-in. The different dataset comprised (i) GTR model or (ii) partitioned CytB according to codon position with substitution model selected by partition finder (K80+G: CytB_1, F81: CytB_2, HKY+G: CytB_3). We searched for rooted and unrooted trees in both approaches. The calculations were conducted on the bPTP websever (http://species.h-its.org/ptp/), with 500,000 MCMC generations and the other parameters remained as default.

- Geometric Morphometrics

Discovery and validation of putative species are best made using an approach that integrates across many data types and analyses (Carsten *et al.,* 2012). As a non-genetic approach geometric morphometric (GM) is a method

efficiently used as species delimitation on taxonomic and systematic studies (Ruane 2015, Mottern & Heraty, 2014). It describes the change of the shape of anatomical landmarks that are systematically repeated in organisms (homologous points) (Corti, 1993). Thus, the configuration of the anatomical landmarks brings with them a diagnostic characteristic to delimit the species or population studied. Despite of that, GM has been poorly used combined with DNA-based approaches to test species boundaries.

Fore wings of male crickets were dissected from alcohol preserved specimens and slide mounted in a drop of water-based gel. The digitalized photos of the wings were all taken in the same position and distance with Axiocam MRc color camera on a Zeiss stereo discovery v20 microscope. To obtain a better image, we used the adjustment of brightness and contrast from the Axiovision software, associated with the microscope.

Twenty-one landmarks were digitalized with TPSdig v2.17 (Rohlf 2013) on each photograph, from which 18 are type 1 (vein crosses) and three are type 2 (maximum curvature).The landmarks were chosen according to the parts of the wing that are involved in the sound production (figure 2).

The 21 landmark configurations representing *Ligypterus* wing shape were analyzed using MorphoJ performing a full Procrustes fit. Covariance matrices of components were computed. We analyzed the relative similarity and discrimination of the eight localities using canonical variates analysis (CVA) and discriminant function analysis (DFA), respectively, of the Procrustes-aligned configurations. This allowed separate analysis of the symmetric and asymmetric components and all pairwise comparisons of localities. The specimens from previous studies (*L. fuscus*,*L. pernambucensis* and *L. linharensis*) were not included in the GM analyses.

Results

Sequence divergence and Bayesian results

Despite the initial dataset comprising *CytB* from 74 specimens of *Ligypterus* we ended up with a final 56 specimens dataset after removing unsuccessful or incomplete sequences. Indeed, given the sensibility of the methods to missing data we could only include sequences that were complete and without ambiguity. For 28S we were able to sequence 46 specimens. Success in sequencing was inversely proportional to the age of the samples, and the unsuccessful sequencing was probably due to the fact that some specimens of the UNESP collection were stored in alcohol 70% for more than 30 years.

The Bayesian results from 28S and *CytB* were very incongruent. Indeed, in the *CytB* tree the specimens were more or less grouped according to the locality (Figure 3), wheras in the 28S tree, the clades had no locality pattern, generally contained specimens from two different origins (Figure 4). We first hypothesized that this could be related to multiple copies of the gene 28S. To eliminate this hypothesis, we conducted a test where we took 6 specimens from different localities and performed 5 PCR reactions from each one, in a total of 30 reactions. If there were different copies, we expected different sequences in the same specimen, but the sequencing did not give this pattern, since each of the 5 sequences taken from the same specimen similar and grouped in a single clade (supplementary material – Figure S1)

ABGD

The results of ABGD analyses are presented in the table 3 and table 4. For CytB sequences with singletons, ABGD estimated between 13 and 10 putative

species in recursive partition. Initial partition showed stability with eight groups in all *P* values. When *P* = 0.005, Murici and Cariacica have two putative species, Linhares and Itamaraju form two different groups each one with its putative species (table 2, figure 5). The next inference (*P*=0.007) the putative species in Murici are gathered in just one. From now on, the increase of p affects Linhares and Itamaraju, assembling their putative species.

For the dataset without singletons, ABGD analysis resulted in 10 to 6 putative species for recursive partition. As the previous dataset, initial partition showed stability with 6 groups in all *P* values. When *P* ranged from 0.01 to 0.02 the major changes were: (i) specimens from Linhares and Itamaraju, after this threshold was considered as a single putative species; (ii) Specimens of Cariacica, instead of separating in two putative species, showed now one single putative specie (table 2, figure 5). The change in gap width from 1.5 to 0.75 did not change the number of putative species in any separate dataset.

GMYC

The results of GMYC analyses are presented in table 5. The Yule tree prior with UCLN clock failed to reject the null model in GMYC (p = 0,157). The number of clusters and putative species were very convergent between all sets of analyses. Across all analyses the proportion of singletons was 21%. The only analysis formed by 10 putative species was the Yule tree prior with strict clock, where samples from Mucuri and Aimorés formed a single putative species as well as samples of Murici and Cariacica. For tree priors Birth/Death or Coalescent with both clocks (strict or UCLN) the number of clusters was 10 and the number of putative species was 13 as follows: (i) each locality represented a putative species, (ii) Cariacica and Murici had two putative species, (iii) the two sequences from L.

fuscus from previous studies formed two different putative specie (table 2, figure 5).

PTP

In PTP the results differed according to the method and substitution model of the input tree (Table 2, Table 6). The differences concerned the number and t configuration of putative species. For Bayesian tree with partitioned dataset we obtain 12 putative species, in which specimens from Cariacica splited into three groups, while those from Aimores and Mucuri formed a single putative species. The same method with GTR dataset resulted in 13 putative species, differing from the previous in the splitting of Itabuna population in two putative species (table x). The ML tree with GTR model led to 11 putative species, with specimens from Mucuri and Aimores forming one putative species. The same happened with specimens from Itamaraju and Linhares. Specimens from Itabuna and Cariacica gaved two putative species each. The partitioned dataset lead in 14 putative species showing Itabuna with 3 and Cariacica with 4 putative species (Table 2, Figure 5).

Geometric morphometrics

Figure 5 shows the CVA of the wings shapes of specimens sampled in different localities (see figure 1– map with localities). The first CVA scatterplot of shape differences between localities shows that Aimorés, Manaus and Cariacica occupy different areas (supplementary material – figure S2). P values of permutation test indicate that specimens from Cariacica (CARM), Manaus (MANM), Itabuna (ITAB) and Linhares (LINM) were divergent (supplementary material – Table S1). The analyses indicated that the specimens from the localities Aimores, Itamaraju and Mucuri had similar wing shape. The results of

discriminant function analysis confirmed the results from CVA. In both methods, Murici wing shape diverged only from Itamaraju and Aimorés (supplementary material – Table S1).

We performed a new CVA and discriminant analyses with Aimorés-Mucuri-Itamaraju forming a single grouping morphoJ (Figure 6). The CVA plot with Aimorés-Mucuri-Itamaraju gathered shows 5 groups (except Murici) with significant p values after permutations tests with different shapes in wing. They were: Manaus (MANM), Cariacica (CARM), Itabuna (ITAB), Linhares (LINM) and Aimorés-Mucuri-Itamaraju (AIMM).

Discussion

Incongruence between nuclear and mitochondrial data

Our mitochondrial and nuclear data are telling different stories. CytB showed relatively clusters more or less grouped according to localities (Figure 3), on the one hand, while 28S shows clusters encompassing two or three localities, on the other hand, without any apparent pattern (Figure 4). Three natural processes are especially noteworthy and could lead to incongruence between gene trees: Multiple gene copies, incomplete lineage sorting and Introgression (Funk & Omland 2003). We eliminated with our test after the first results of 28S the possibility of multiple copies. Incomplete lineage sorting and introgression may be natural processes behind our incongruence pattern between 28S and CytB.

After speciation, genetic drift and new alleles being formed by mutation will result in daughter species becoming increasingly distant from each other and from their ancestor. However, if the time since speciation is too short, the common ancestral alleles remain in both species in the some loci, and incomplete

lineage sorting will be observed. Consequently, in species with recent speciation, the phylogenetic hypothesis doesn't reflect the species tree. Given the haploidy, maternal inheritance and N_e (effective population size) of mitochondrial DNA, it is expected that the progress of lineage sorting occurs more slowly in nuclear genes when compared to mitochondrial genome (Funk & Omland 2003).

Alleles from one species may penetrate the gene pool of another through interspecific mating and the subsequent backcrossing of hybrids into parental populations, a process known as introgression (Funk & Omland 2003). The rates of mitochondrial and nuclear introgression often differ, with some taxa showing biases for mitochondrial introgression and others for nuclear introgression. The cases where nuclear introgression exceed mitochondrial includes (i) when the female is the heterogametic sex (Patten *et al.,* 2015), (ii) and when there is a sex difference in dispersal rates (Petit & Excoffier 2009).

When the female is the heterogametic sex, the fertility and viability is reduced in female hybrids restricting mitochondrial gene flows (Funk & Omland 2003, Patten *et al.,* 2015). The primitive sex determination system of crickets is the X0/XX, despite the continuous emergence of new patterns (Castillo *et al.,* 2010, Zefa *et al.,* 2014). No previous cytogenetic study about *Ligypterus* was found, consequently, the sex determinations system in this genus remains unclear.

Petit & Excoffier (2009) made the assumption that once one species enters in contact with another, if a given gene experience high intraspecific gene flow, lower are the chances of introgression within this marker. Females and males have different dispersal behavior within species. In systems were exist a female biased dispersal, the mtDNA is more conserved, protected by high gene flows. On the other hand, the nuclear DNA suffers more with introgression. Previous studies about dispersal behavior in *Ligypterus* are unknown to us.

Further effort to discover these two important characteristic (cytogenetic pattern and dispersal behavior) are crucial to answer the incongruence in nuclear and mitochondrial genes in *Ligypterus*.

How many* Ligypterus *species are there?

Ligypterus is a taxonomically difficult group to work, since the species are at the same time morphologically very similar while presenting interspecific variation. Classical criteria used for cricket taxonomy, such as analysis of male and female genitalia, fail to separate closely related species when a dense sampling is available. This is the problem that accompanies many cryptic species, which real diversity should then be considered with different approaches together with morphological analyses. In this context, the use of species delimitation methods based on DNA are seen as a promising tool to assess species boundaries. The results of our investigations of *Ligypterus* diversity were remarkably congruent between the methods that we used. Considering all the analyses in a conservative way estimates range from 5 to 10 putative species for the sampled localities (GMYC Yule/strict clock= 10 putative species; PTP ML with GTR model = 11; ABGD with singletons *P* from 0.0136 to 0.0189 = 10, and Geometric morphometrics = 5)

Compared with molecular approaches, we conducted our analysis of geometric morphometric with a very limited dataset. In all molecular approaches Murici resulted in a distinct cluster. Based on GMYC, the specimens from Murici showed a high diversity with two putative species. Despite of that, in GM, the shape of Murici was only different from Aimorés group. Sampling in Murici was very poor, encompassing only two specimens. This probably influenced the results of Murici in canonical and discriminant analysis. It was also not possible to

include in the GM dataset the samples from previous studies, i.e. material from previously described species, which were partly available in the DNA delimitation methods.

Our integrative approach in *Ligypterus* diversity helped us to identify a number of "stable" putative species, which were supported by different lines of evidence (genetic and non-genetic). Based on our analyses, the specimens from Itabuna, Cariacica, Murici and Manaus could be unambiguously distinguished from the specimens examined. We have in our analysis sequences of three recognized *Ligypterus* species: *Ligypterus linharensis*, *Ligypterus pernambucensis* and *Ligypterus fuscus*. Among these, only the sequences of *L. linharensis* belonged in a cluster, together with the specimens from Linhares. The other remained isolated, indicating possibly new species in the localities sampled in this study. The other two described species that were not present in our dataset are *L. heydeni* and *L. belmontensis.* The first one was described based on the female copulatory papilla, with very poor diagnostic characters and no accurate geographical distribution (Type locality = Brazil; Robillard & Desutter-Grandcolas 2005); it will thus probably never be possible to attribute this name to any specimen of recently collected material. The specie *L. belmontensis* is geographically very close to Itamaraju, and future extractions must be done to identify where this specie will cluster in our phylogenetic hypothesis. Given that *Ligypterus* has five species currently described, our estimates indicate that the real diversity of the genus may be twice this number.

The localities from Cariacica, Murici and Itabuna deserve special attention as interesting localities for future collections, since more than one putative species of *Ligypterus* may coexist in these places. Are there sympatric closely related species with different micro-habitats or calling signal keeping them separated?

Our results showed, on the other hand, conflicts between the methods in Linhares, Itamaraju, Aimorés and Mucuri. These localities need additional morphological and molecular information to confirm their diversity status.

The use of a variety of tools to access the boundary between species proved to be effective and of great help in may recent studies (Ceccarelli *et al.,* 2012, Le Ru *et al.,* 2014, Dumas *et al.,* 2015, Schwarzfeld & Sperling 2015,). However, these tools cannot be used blindly, it should be seen as exploratory methods that allow us to identify putative species cluster that requires an integrative taxonomic approach. In the groups poorly delimitated, a fine scale morphological analysis can sometimes help to solve the conflicts, or it could reveal that differences in species diversify. On the other hand, clusters that are consistently defined by these tools are strong candidates to species, and may be examined for morphological confirmation detail in order to refute or not the delimitation method based on DNA.

Biogeographical distribution

Nowadays, *Ligypterus* is known mainly from South-eastern coastal Brazil, with a few records from French Guiana and Manaus (Fig. 1). Along the coast, Cariacica is the most southern locality where we could find specimens of this genus, which is situated below Doce River. Still in the coast, the upper limit that we can collect *Ligypterus* is Murici, the only locality above São Francisco River. In between, the coast is also crossed by Jequitinhonha River. It means that our sampled localities are highly divided by rivers along the coast: upper from Jequitinhonha until São Francisco River, we have Itabuna. Below Jequitinhonha until Doce, we have Mucuri, Aimores, Itamaraju and Linhares. Given this, the recent coastal distribution of *Ligypterus* appears to have been limited by the major

rivers in Brazil (Doce, Jequitinhonha and São Francisco), suggesting a riverine-barrier hypothesis to explain the recently diverged putative species in the *Ligypterus* complex (Moritz *et al.,* 2000).

The mid-Miocene climatic optimum was an episode between 17 and 15 Ma marked by a wet period followed by abrupt changes in temperature. This short term environmental changes may have led to forest expansion and contraction, leading to isolation of forest-adapted species (Hinojosa 2005, Fouquet *et al.,* 2014). Past connections between Atlantic Forest and Amazon basin resulted in disjunctive distributions between sister clades of extant species (Batalha-Filho *et al.,* 2013, Sobral-Souza *et al.,* 2015). The beginning of divergence within *Ligypterus* could thus be a result of abrupt changes of temperature subsequent to the mid-Miocene climatic optimum. *L. fuscus* from French Guiana and the new putative specie from Manaus may thus have originated during these climatic events.

Conclusion

Based on morphology, Robillard & Desutter-Grandcolas (2005) estimated a diversity of 5 species of *Ligypterus*. This study indicates that the true number of this Neotropical cricket is considerably higher and with more complete sampling may double that estimate. This constitutes another case study that underlines the interest of fast species discovery procedures. Allied to taxonomy, it constitutes a new line of evidence in specie discovery toward a better knowledge of planet diversity. Specifically for *Ligypterus*, it fuels the curiosity of which variables may influence the pattern of sympatric distribution of closely related species in

Cariacica and maybe Murici. Given the recent association of this genus with high frequency signal (Robillard *et al.,* 2015), further studies relying on bioacoustics and reproductive behavior will be necessary to acknowledged that.

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Figure Legends

Figure 1. Collecting areas for this study and species from previous studies are indicated as dots on the map. We also present the three important rivers in Brazil, Rio Doce, Rio Jequitinhonha and Rio São Francisco.

Figure 2. Twenty one landmarks on *Ligypterus* wing used in this study.

Figure 3. Phylogenetic relationship of *Ligypterus* inferred by bayesian analyses based on CytB. The different localities of this study are represented: Itab = Itabuna, Mur = Murici, Aim = Aimorés, Muc = Mucuri, Itam = Itamaraju, Lin = Linhares, Car = Cariacica, Man = Manaus. In the dataset is also included species described from the museum d'histoire naturelle: Lper = *Ligypterus pernambucensis*, Lf2 = *Ligypterus linharensis*, LfuNo2 and LfuPa = *Ligypterus fuscus*. As outgroup we used *Ponca Venosa.*

Figure 4. Phylogenetic relationship of *Ligypterus* inferred by bayesian analyses based on 28S. The different localities of this study are represented: Itab = Itabuna, Mur = Murici, Aim = Aimorés, Muc = Mucuri, Itam = Itamaraju, Lin = Linhares, Car = Cariacica, Man = Manaus. As outgroup we used *Eneoptera sp.*

Figure 5. Putative species cluster corresponding to: GMYC analyses based on a Y/st = strict clock and Yule model (column 1) or O= all the other analyses (strict clock with Coalescent or B/D model; UCLN clock with Yule or B/D or Coalescent model) (column 2); PTP analysis where 1 = Bayesian method and partitioned dataset (column 3), 2 = Bayesian method with GTR (column 4), 3 = Maximum likelihood method with GTR (column 5) and 4 = Maximum likelihood with partitioned dataset (column 6); ABGD analyses which A = initial partition from dataset with singletons (column 7), B = recursive partition (P=0,00136) from dataset with singletons (column 8), C = initial partition from dataset without singletons (column 9) and D = = recursive partition (P=0,00136) from dataset without singletons (column 9). In a given column, each color represents a putative specie.

Figure 6. Canonical variates analyses of wing shape variation using covariance matrices polled with localities after the grouping of Aimorés-Itamaraju-Mucuri.

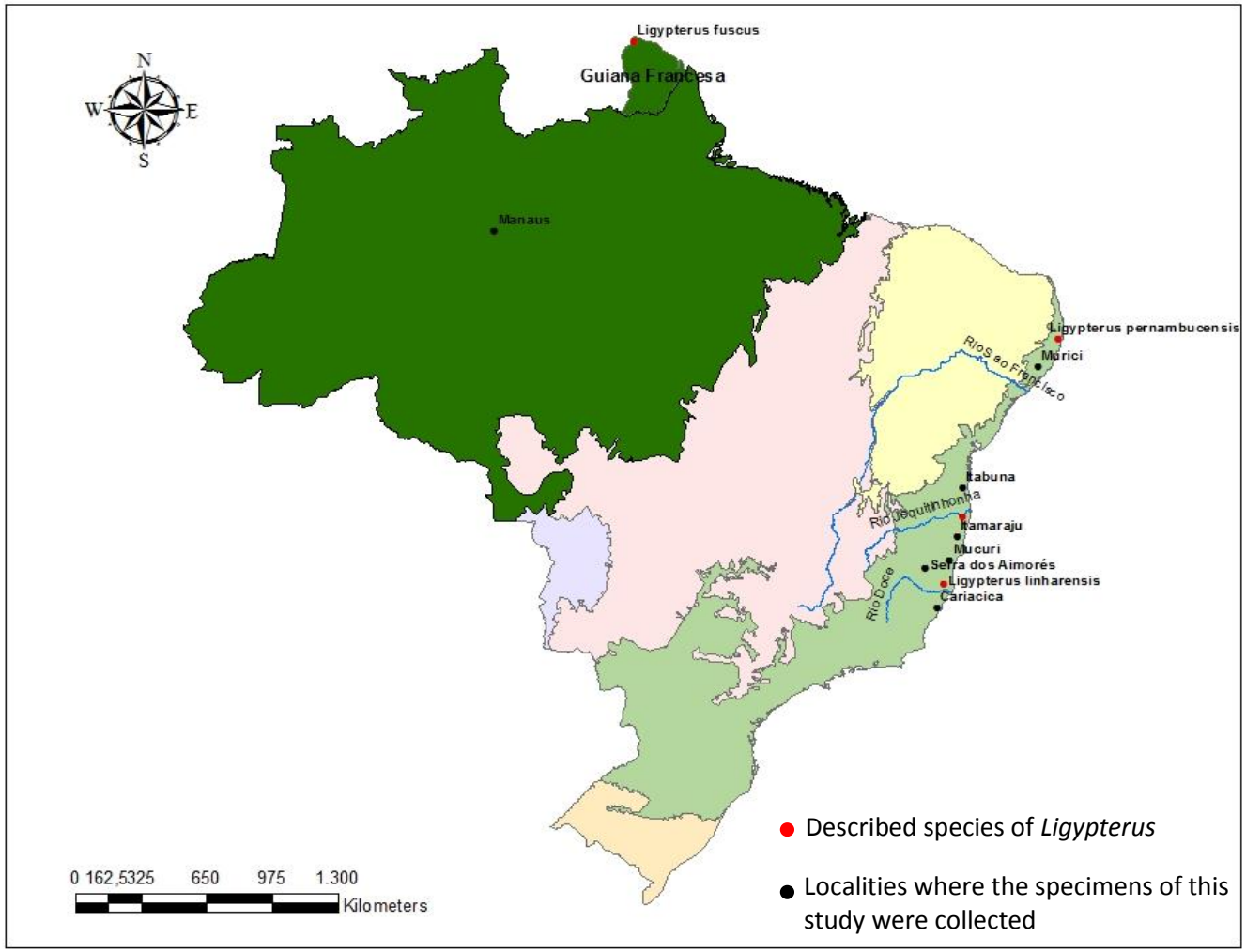

Ligypterus fuscus
Guiana Francesa
N
W
E
S
Manaus
Ligypterus pernambucensis
Morici
Rio São Francisco
Itabuna
Rio Jequitinhonha
Itamaraju
Mucuri
Serra dos Aimorés
Ligypterus linharensis
Cariacica
Rio Doce
0 162,5325 650 975 1.300
Kilometers
Described species of *Ligypterus*
Localities where the specimens of this study were collected

Figure 1

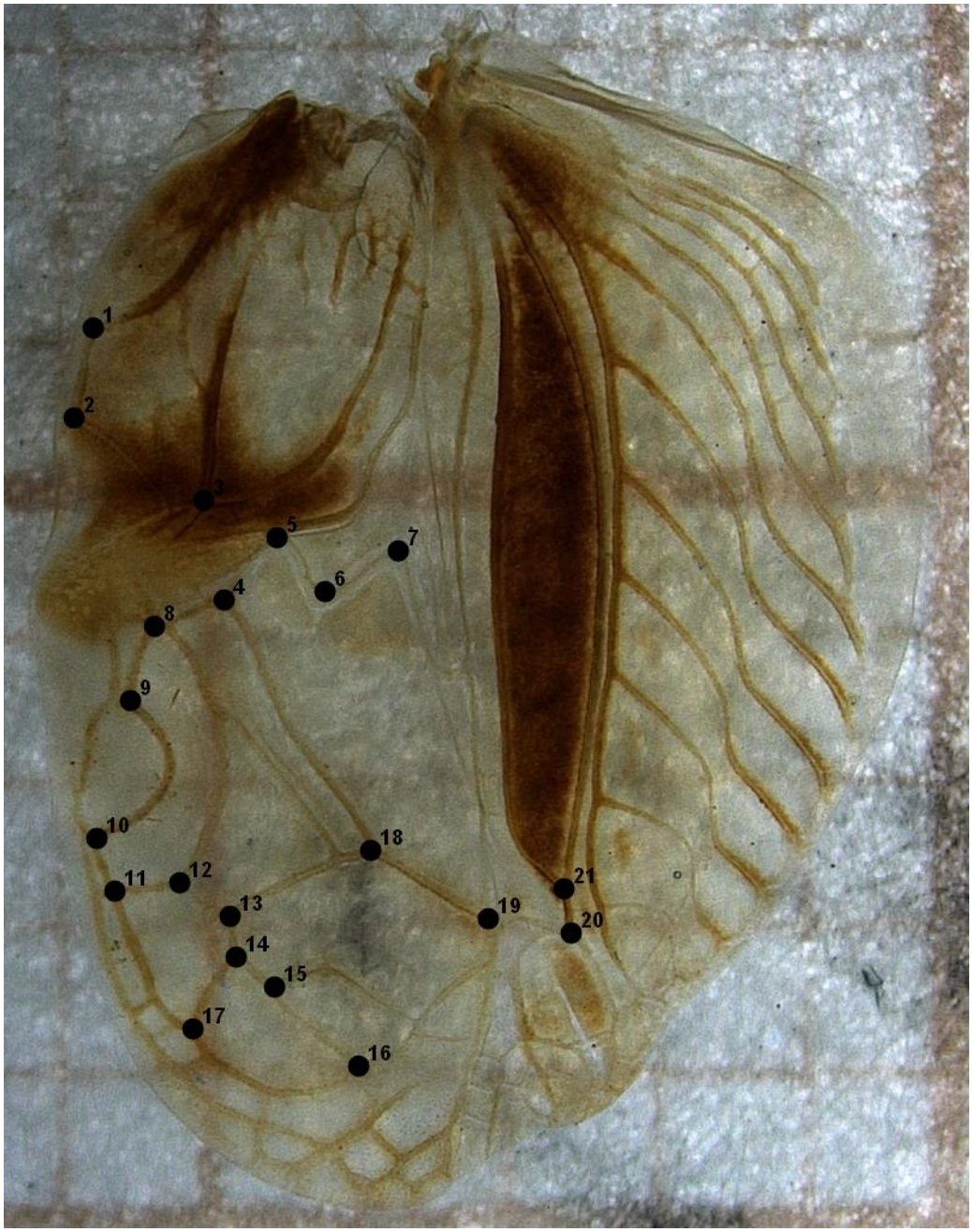
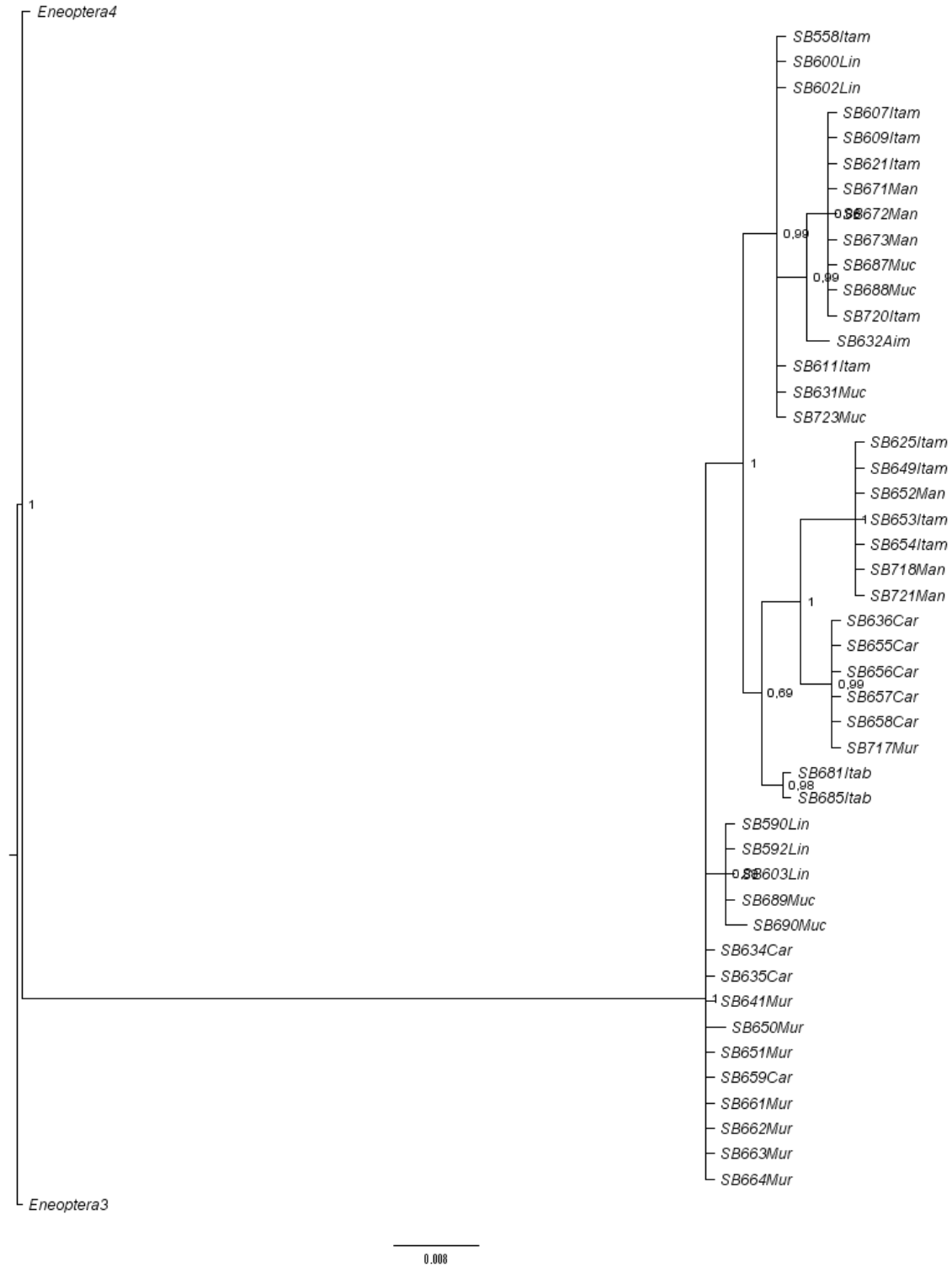


Figure 2


Eneoptera4
SB558Itam
SB600Lin
SB602Lin
SB607Itam
SB609Itam
SB621Itam
SB671Man
SB672Man
SB673Man
SB687Muc
SB688Muc
SB720Itam
SB632Aim
SB611Itam
SB631Muc
SB723Muc
SB625Itam
SB649Itam
SB652Man
SB653Itam
SB654Itam
SB718Man
SB721Man
SB636Car
SB655Car
SB656Car
SB657Car
SB658Car
SB717Mur
SB681Itab
SB685Itab
SB590Lin
SB592Lin
SB603Lin
SB689Muc
SB690Muc
SB634Car
SB635Car
SB641Mur
SB650Mur
SB651Mur
SB659Car
SB661Mur
SB662Mur
SB663Mur
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Eneoptera3
0,99
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1
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0,69
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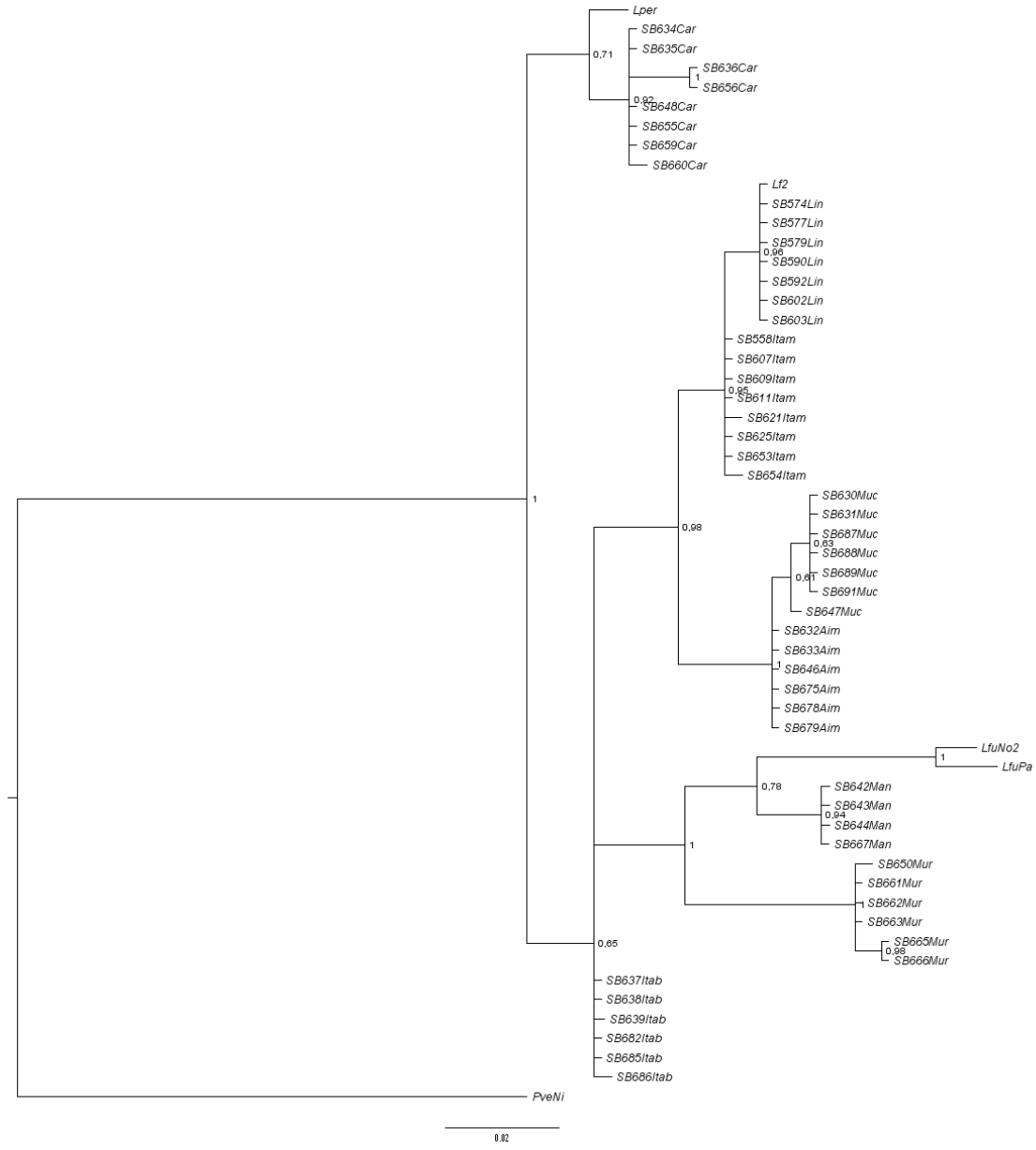
0,98
1
0.008

Figure 3


Lper
SB634Car
SB635Car
SB636Car
SB656Car
SB648Car
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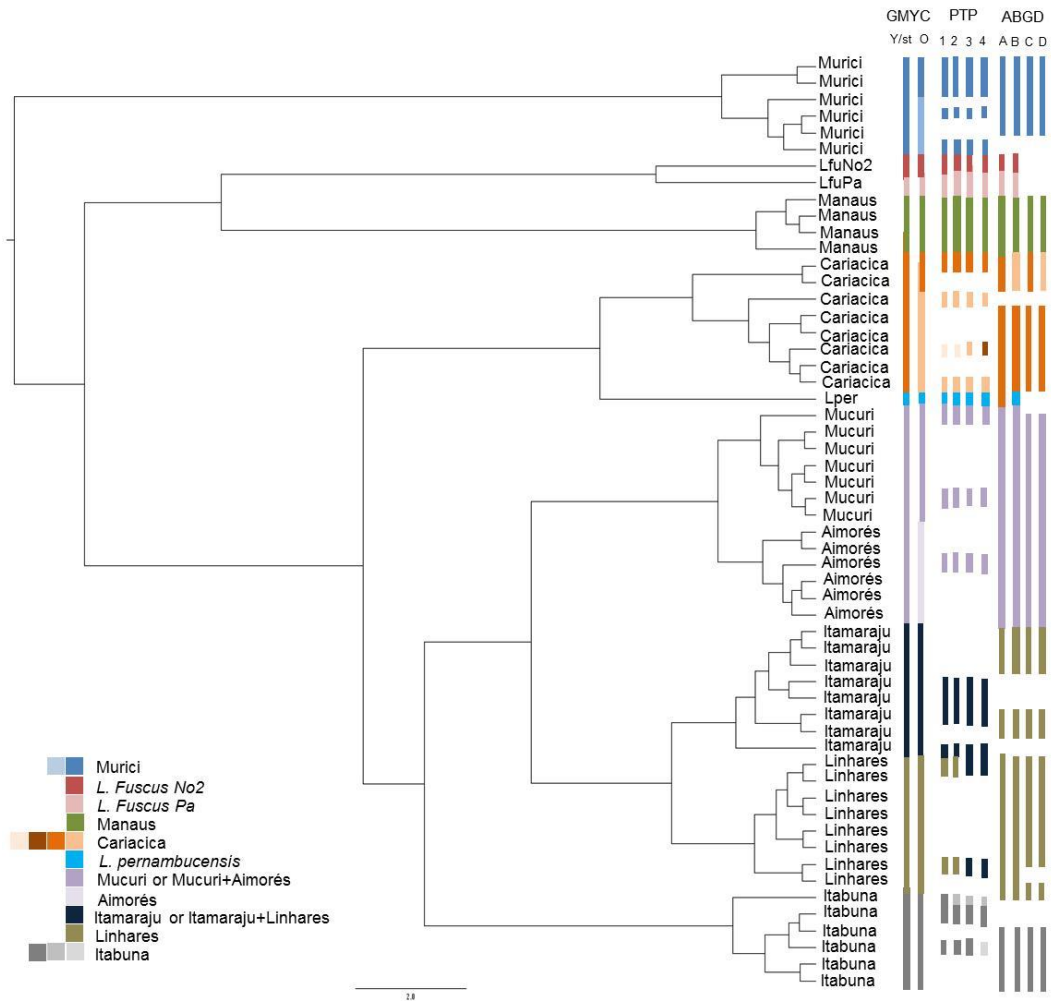
SB659Car
SB660Car
Lf2
SB574Lin
SB577Lin
SB579Lin
SB590Lin
SB592Lin
SB602Lin
SB603Lin
SB558Itam
SB607Itam
SB609Itam
SB611Itam
SB621Itam
SB625Itam
SB653Itam
SB654Itam
SB630Muc
SB631Muc
SB687Muc
SB688Muc
SB689Muc
SB691Muc
SB647Muc
SB632Aim
SB633Aim
SB646Aim
SB675Aim
SB678Aim
SB679Aim
LfuNo2
LfuPa
SB642Man
SB643Man
SB644Man
SB667Man
SB650Mur
SB661Mur
SB662Mur
SB663Mur
SB665Mur
SB666Mur
SB637Itab
SB638Itab
SB639Itab
SB682Itab
SB685Itab
SB686Itab
PveNi
0.71
0.92
1
0.96
0.85
1
0.98
0.63
0.61
1
0.78
1
0.94
1
1
0.65
0.98
0.02

Figure 4


GMYC
Y/st O
PTP
1 2 3 4
ABGD
A B C D
Murici
Murici
Murici
Murici
Murici
Murici
LfuNo2
LfuPa
Manaus
Manaus
Manaus
Manaus
Cariacica
Cariacica
Cariacica
Cariacica
Cariacica
Cariacica
Cariacica
Cariacica
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Mucuri
Mucuri
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Aimorés
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Itabuna
Itabuna
Itabuna
Itabuna
Itabuna
Itabuna
Murici
L. Fuscus No2
L. Fuscus Pa
Manaus
Cariacica
L. pernambucensis
Mucuri or Mucuri+Aimorés
Aimorés
Itamaraju or Itamaraju+Linhares
Linhares
Itabuna

Figure 5

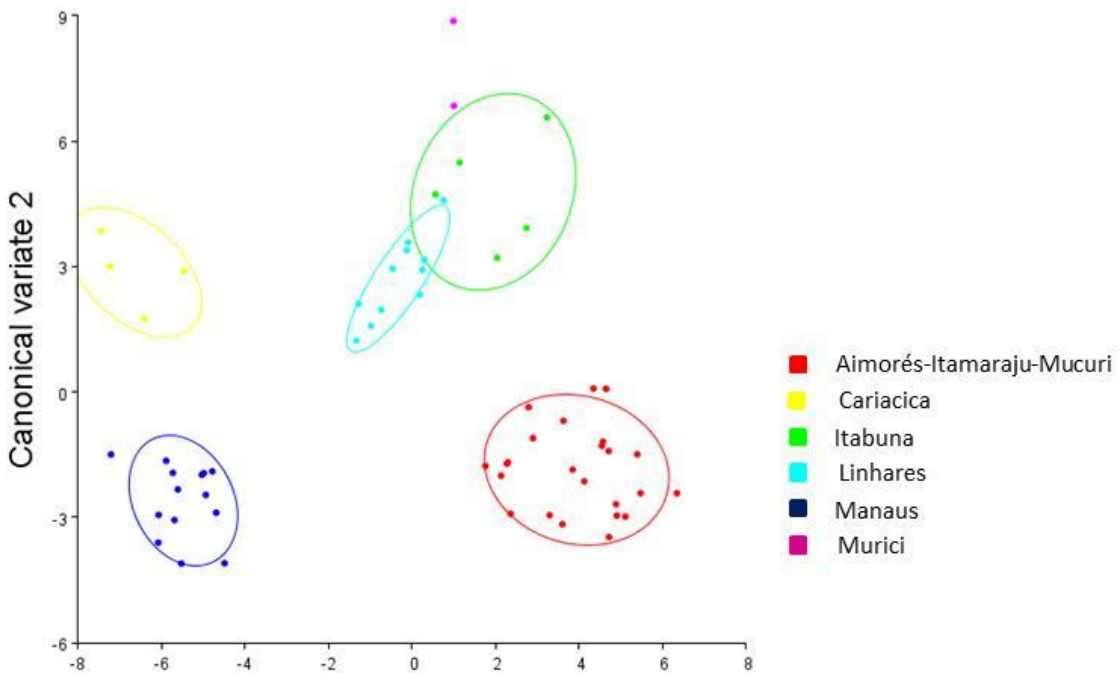
Canonical variate 2
9
6
3
0
-3
-6
-8
-6
-4
-2
0
2
4
6
8
Aimorés-Itamaraju-Mucuri
Cariacica
Itabuna
Linhares
Manaus
Murici

Figure 6

Table Legends

Table 1. Sequence variation in each dataset examined. N = number of sequences in the data set; Bp = number of base pairs; VS = variable sites.

Table 2. Putative number of species recovered of *Ligypterus* by all methods according to the locality or sequence from previous studies (*L. fuscus*, *L. pernambucensis*). *:Prior intraspecific divergence; BD/Strict: tree prior Birth/Death with strict clock; BD/UCLN: tree prior Birth/Death with uncorrelated lognormal relaxed clock; Co strict: tree prior coalescent with strict clock; Co UCLN: tree prior coalescent with uncorrelated lognormal relaxed clock; Yule strict: tree prior yule with strict clock; Yule UCLN: Tree prior yule with uncorrelated lognormal relaxed clock; MLP: maximum likelihood tree with partitioned dataset; ML: maximum likelihood tree with GTR model; BI: Bayesian tree with GTR model; BIP: Bayesian tree with partitioned dataset; ns = $p>0.01$

Table 3. Putative species recovered from Cytb dataset with singletons of *Ligypterus* by ABGD according to gap width (0.75 or 1.5) and prior intraspecific divergence (*P*). *:Prior intraspecific divergence; I:Initial partition; R: recursive partition.

Table 4. Putative species recovered from Cytb dataset without singletons of Ligypterus by ABGD according to gap width (0.75 or 1.5) and prior intraspecific divergence (*P*). *:Prior intraspecific divergence; I:Initial partition; R: recursive partition.

Table 5. Number of GMYC putative species recovered and outputs obtained from the single-threshold GMYC analyses performed for CytB. Ultrametric tree reconstructions with the following parameters: Tree prior: Yule, Birth/Death and Coalescent; Clock: Strict or UCLN (uncorrelated lognormal relaxed clock model; NC = number of clusters (GMYC ‘‘species’’ with more than one individual); PS = number of GMYC putative species; CI = confidence intervals of GMYC putative species; L-null = likelihood of null model; L-GMYC = likelihood of GMYC model; LRT = likelihood ratio with significance indicated by an asterisk. * $p < 0.01$.

Table 6. Number of GMYC putative species recovered by PTP according to substitution model and phylogenetic method. Bayesian: Bayesian method of phylogenetic inference; ML: Maximum likelihood method of phylogenetic inference; GTR: generalized time reversible (substitution model); Partitioned: CytB partitioned by codon position and with substitution model selected by partition finder and Iq-tree automatically.

	N	BP	VS
$CytB_{sing}$	48	346	64
$CytB_{nosing}$	44	346	44
GMYC	56	346	65
PTP	28	348	65
28S	46	400	16

Table 1

Locality/sequence from previous studies	Morphometrics	ABGD (singletons)		ABGD (no singletons)			GMYC						PTP			
		0.007*	0.0097*	0.0070*	0.0097*	0.0189*	BD/Strict	B/D UCLN	Co strict	Co UCLN	Yule strict	Yule UCLN	MLP	ML	BI	BIP
Cariacica	1	2	2	2	2	1	2	2	2	2	1	2	4	2	3	3
Manaus	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Itamaraju	0	1	0	1	0	0	1	1	1	1	1	1	0	0	1	1
Murici	0	1	1	1	1	1	2	2	2	2	1	2	1	1	1	1
L. Fuscus	0	2	2	--	--	--	2	2	2	2	2	2	2	2	2	2
Mucuri	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0
Aimores	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0
Itabuna	1	1	1	1	1	1	1	1	1	1	1	1	3	2	2	1
L. pernambucensis	0	1	1	--	--	--	1	1	1	1	1	1	1	1	1	1
Linhares	1	1	0	1	0	0	1	1	1	1	1	1	0	0	1	1
Mucuri+Aimores	0	1	1	1	1	1	0	0	0	0	1	0	1	1	1	1
Itamaraju+Linhares	0	0	1	0	1	1	0	0	0	0	0	0	1	1	0	0
Itam+Muc+Aim	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	**5**	**11**	**10**	**8**	**7**	**6**	**13**	**13**	**13**	**13**	**10**	**ns**	**14**	**11**	**13**	**12**

* Prior intraspecific divergence in ABGD

Table 2

WITH SINGLETON								
Dataset	**0.0050***		**0.0097***		**0.0136***		**0.0189***	
	I	**R**	**I**	**R**	**I**	**R**	**I**	**R**
Gap width 0.75	8	13	8	11	8	10	8	10
	I	**R**	**I**	**R**	**I**	**R**	**I**	**R**
Gap width 1.5	8	13	8	11	8	10	8	10

Table 3

WITHOUT SINGLETON												
Dataset	**0.0050***		**0.0070***		**0.0097***		**0.0136***		**0.0189***		**0.0264***	
	I	**R**	**I**	**R**	**I**	**R**	**I**	**R**	**I**	**R**	**I**	**R**
Gap width 0.75	6	10	6	8	6	7	6	7	6	6	6	6
	I	**R**	**I**	**R**	**I**	**R**	**I**	**R**	**I**	**R**	**I**	**R**
Gap width 1.5	6	10	6	8	6	7	6	7	6	6	6	6

Table 4

Tree prior	Clock	NC	PS	CI	L-null	L-GMYC	LRT
Yule	Strict	7	10	6-17	92.71943	97.03291	0.013*
	UCLN	7	7	1-25	60.2005	62.05152	NS
Birth/Death	Strict	10	13	9-24	128.2716	134.4027	0.0021*
	UCLN	10	13	9-22	126.7578	132.6677	0.002*
Coalescent	Strict	10	13	8-24	116.282	121.6775	0.004*
	UCLN	10	13	8-27	114.5994	118.8348	0.014*

Table 5

	Bayesian	ML
GTR	13	11
Partitioned	12	14

Table 6

Appendices

a) Test to eliminate the assumption of multiple copies of 28S in *Ligypterus*

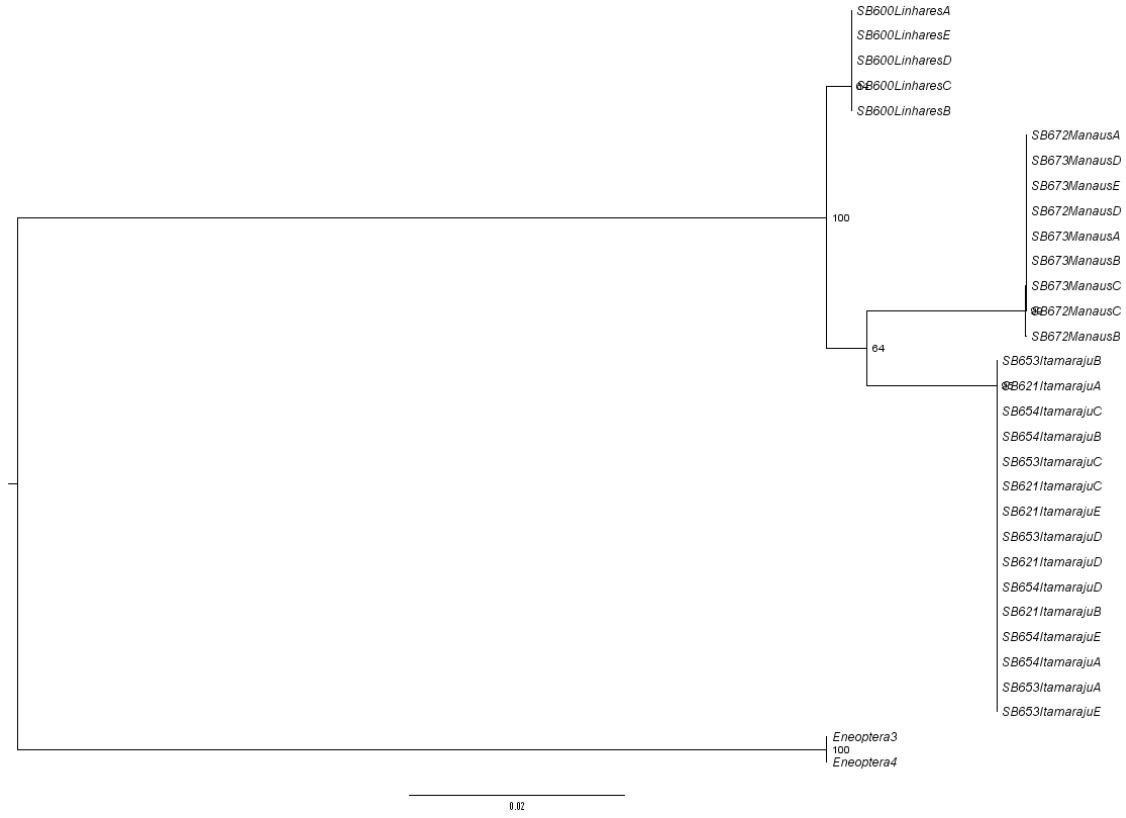

Figure S1. Phylogenetic relationship of *Ligypterus* inferred by Bayesian analyses based on 28S. This analysis consisted in 6 selected specimens from each locality. In each individual we performed five PCR reactions with the 28S marker, resulting in 30 PCR reactions in total. With this test, the assumption of multiple copies of 28S in Ligypterus were eliminated. The different localities of this study are represented: Itab = Itabuna, Mur = Murici, Aim = Aimorés, Muc = Mucuri, Itam = Itamaraju, Lin = Linhares, Car = Cariacica, Man = Manaus. As outgroup we used *Eneoptera sp.*

b) Morphometric Geometric details

Table S1. Procrustes values for canonical variates analyses

	Cariacica	Itabuna	Itamaraju	Linhares	Manaus	Mucuri	Murici	Aimorés
Cariacica		0.0398	0.0501	0.0278	0.0278	0.0384	0.0308	0.0521
Itabuna	0.0079		0.0364	0.027	0.0347	0.0335	0.0238	0.0376
Itamaraju	<.0001	<.0001		0.0293	0.0358	0.0189	0.0313	0.0234
Linhares	0.0081	0.003	<.0001		0.0258	0.022	0.0236	0.0322
Manaus	0.0073	0.0002	<.0001	0.0007		0.0262	0.0229	0.0404
Mucuri	0.0011	0.0009	0.027	0.0109	0.0001		0.0257	0.0249
Murici	0.2711	0.4729	0.0201	0.518	0.5387	0.2914		0.0398
Aimorés	0.0008	<.0001	0.0826	0.0024	0.0008	0.0816	0.0324	

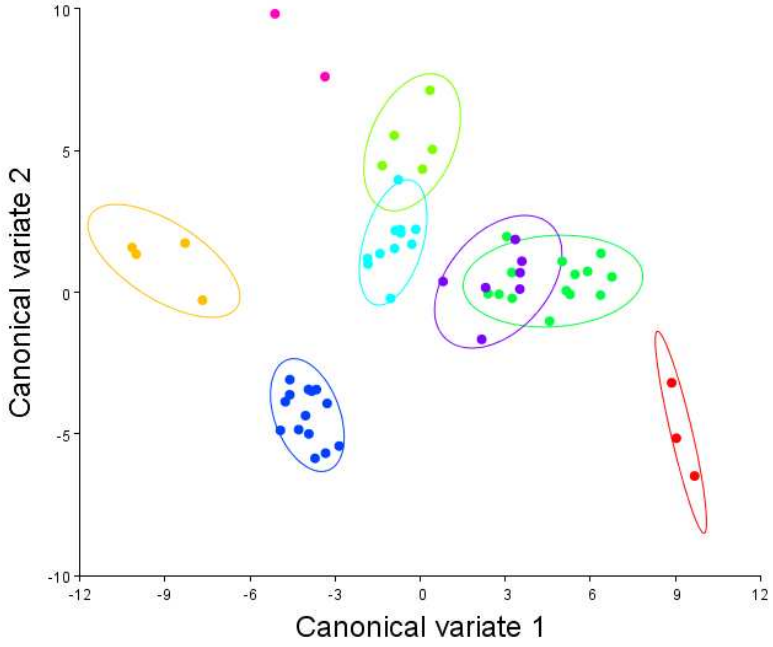

Figure S2. Canonical variates analyses of wing shape variation using covariance matrices polled with localities before the grouping of Aimorés-Itamaraju-Mucuri.

CHAPTER FOUR

Gnominthus gen. nov., a new genus of crickets endemic to Papua New Guinea with novel acoustic and behavioral diversity (Insecta, Orthoptera, Gryllidae, Eneopterinae)

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Gnominthus gen. nov., a new genus of crickets endemic to Papua New Guinea with novel acoustic and behavioral diversity (Insecta, Orthoptera, Gryllidae, Eneopterinae)

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ABSTRACT

The present study describes *Gnominthus*, a new genus of Eneopterinae crickets from New Guinea Island (Papua New Guinea), which belongs to the tribe Lebinthini. Descriptions focus on general morphology, male and female genitalia, and forewing venation. Bioacoustical analyses of the calling song and the description of the mating behavior are also provided. The novelties found here increase the idea hypothesized before that the Lebinthini may represent a very diverse group in terms of shapes, behaviors and acoustic signals.

1. Introduction

The cricket subfamily Eneopterinae (Gryllidae) is mostly diversified in the Western Pacific region (Otte, 2007; Robillard, 2009, 2011; Robillard et al., 2014). A close look at the fragmentary knowledge from this region suggests that the greatest part of this diversity is yet likely to be discovered and formally described. This is the case for the eneopterine fauna of New Guinea Island, which is known only through a few briefly described species (e.g., Brunner von Wattenwyl, 1898; Chopard, 1931; Bhowmik, (1979) 1981; Otte, 2007). The eneopterine species from New Guinea all belong to the tribe Lebinthini Robillard, 2004, which are the first crickets known to communicate with high frequencies (>10 kHz), and in some species with ultrasonic signals (26–28 kHz) (Robillard and Desutter- Grandcolas, 2004a; Robillard et al., 2007, 2013). Recent results supported the hypothesis that high-frequency songs may have evolved in a stepwise manner rather than only by a progressive increase of the ancestral dominant frequency (Robillard et al., 2013). The basis for this resides in the pre-existing harmonic structure of songs generated by the resonant properties of wing radiators. Other recent studies also suggested that the Lebinthini not only diversified through multiplication of species numbers as a possible consequence of this acoustic innovation, but that they also diversified upon the way that these crickets produce and use high frequencies (Robillard et al., 2015). As a consequence, it is important to document the bioacoustics of these species in parallel to the taxonomical studies documenting new species and new genera.

During a recent field expedition in Papua New Guinea, we collected specimens corresponding to a new genus and a new species of the brachypterous Lebinthini clade. This new taxon showed original morphological traits, but also new combinations of acoustic features never observed in this group, as well as original mating behaviors. In the present study we describe the genus *Gnominthus* gen. nov. and the species *G. baitabagus* n. sp. focusing on general morphology, coloration, genitalia and forewing venation in both sexes. We also describe the calling song and analyze the stridulatory mechanism at the origin of the spectral pattern of the song. Finally, we describe the mating behavioral sequence and its particularities in comparison with existing data about other lebinthine species.

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2. Materials and methods

2.1. Material

The crickets studied herein were collected during an expedition in Papua New Guinea in Oct–Dec 2012, organized by Our Plant Reviewed (Niugini 2012, MNHN-Pronatura International). Specimens were collected by sight only, at night, and deposited in the collections of the Muséum national d'Histoire naturelle, Paris (MNHN). Square brackets in lists of materials are used for additional information not mentioned on specimen labels or translated from French labels.

2.2. Observations and morphological analysis

Morphological description follows the pattern and terminology of recent publications (e.g., Robillard, 2010; Robillard et al., 2014). Direct observations and dissections have been made using a binocular microscope Leica MZ16 at magnifications up to 115×. Male tegminal veins and cells follow terminology by Ragge (1955) and Robillard and Desutter-Grandcolas (2004b). Male and female genitalia have been dissected in softened or fresh specimens by cutting the membranes between the paraprocts and the subgenital plate, or between the ovipositor and the subgenital plate respectively; they have been cleaned with cold KOH and then kept in glycerine in vials pinned under specimens. Male genitalia are named according to Desutter (1987), modified in Desutter- Grandcolas (2003) and Robillard and Desutter-Grandcolas (2004b). Photographs of male genitalia were obtained using an AmScope MU1000 digital camera (www.Amscope.com). Genitalia were tinted with a drop of Punktol product (S. Hugel, comm. pers.).

2.3. Acoustic study

The basic cricket song terminology follows Ragge and Reynolds (1998): one song unit is called a syllable and corresponds to one opening–closing cycle of the male forewings; a group of syllables forming a cricket call is named an echeme.

Gnominthus baitabagus has been recorded in the laboratory. The recordings were made with a modified Condenser Microphone Capsule CM16 (Avisoft Bioacoustics, Berlin), with a relatively flat frequency response from 3 to 150 kHz (R. Specht pers. comm.), using Avisoft Triggering Harddisk Recorder version 2.97 and a 8-Pre MOTU sound card at a sampling frequency of 96 k-samples s^{-1} (16 bit). Song features were measured using the automatic commands under Avisoft-SASLab Pro version 4.40 (Specht, 2008). All recording files are deposited in the Sound Library of the MNHN.

2.4. Study of stridulation

We estimated wing velocities during sound production with the method described in Robillard et al. (2015): We compared wing velocity measurements with instantaneous velocity in order to determine whether the dominant frequency of the song corresponds to the natural fundamental frequency or to a higher harmonic. For such comparison, wing velocity is calculated as the length of functional region of the file divided by the whole FW closing time (=syllable duration), and instantaneous velocity is calculated by dividing the average inter-tooth distance by the time separating two subsequent waveforms in the song for a candidate value of fundamental frequency (=1/frequency value). The instantaneous velocity that most closely matches the wing velocity measurement is then supposed to correspond to the tooth-strike rate (TSR). Irrespective of the relative energy of each peak, if the first peak of the spectrum matches the tooth impact, it means that each functional file tooth produces one elementary waveform of the song; the dominant frequency is then directly produced by the stridulatory mechanism, as described for Gryllinae for example (e.g., Montealegre-Z. et al., 2009). However, if the wing velocity better matches a higher instantaneous velocity, it suggests that more than one waveform is produced per tooth strike, because of the harmonic vibration of the wings, as suggested for some other eneopterine crickets (Robillard et al., 2007, 2013). Also, when the main peak of song frequency corresponds to a second or third harmonic, there is generally a corresponding modulation of the sound wave, so that every third or fourth sound wave is slightly higher in amplitude, followed by some dampening.

2.5. Behavioral study

Cricket nymphs (generation F0) were raised in the laboratory for behavioral studies. Specimens of F1, F2 and F3 generations were used for trials. Virgin individuals of both sexes were separated as sub-adults. After approximately two weeks of maturation after final moult, we placed a couple of crickets in a glass container with a mirror background for observation under normal artificial room light. The complete sequences of reproductive behavior of four couples were recorded. Video recordings were done using a Sony Handycam HDR-HC3 video recorder. Behavioral sequences were analyzed using the program JWatcher version 1.0 (Blumstein et al., 2000–2012).

2.6. Abbreviations

General morphology:

I, II, III front, median, hind, respectively (femora, legs, tibiae); F: femora; FW: forewing; Tarsomere III-1: basal segment of hind leg tarsomere; T: tibiae.

Male genitalia:

ect ap: ectophallic apodeme; ect arc: ectophallic arc; ect f: ectophallic fold; ect lat exp: ectophallic lateral expansion; end ap: endophallic apodeme; end s: endophallic sclerite; pse p: pseudepiphallic paramere.

Tegminal venation:

1A-4A: first to fourth anal veins; CuA: anterior cubitus; CuA1, CuA2, . . .: first, second, . . . bifurcations of CuA; CuP: posterior cubitus; M: median vein; Sc: subcostal vein; R: radial vein; di, diagonal; ob, oblique veins, c1-3: first to third cells of C alignment; d1 cell (mirror): first cell(s) of D alignment; d2: second cell of D alignment; e1: first cell of E alignment.

Morphological measurements:

FIIIL: length of hind femora; FIIIW: width of hind femora; FWL: forewing length; FWW: forewing width (at the level of maximal width); Ias: inner spines on TIII dorsal side, above the spurs; Ibs: inner spines on TIII dorsal side, between the spurs; Oas: outer spines on TIII dorsal side, above the spurs; Obs: outer spines on TIII dorsal side, between the spurs; OL: ovipositor length; PronL: pronotum length; PronW: pronotum width; TIIIL: length of hind tibiae; TaIIIs: spines on outer edge of third hind tarsomere, not including the apical spine.

Acoustics:

fd: dominant frequency; TSR: tooth-strike rate.

3. Taxonomy

3.1. Generic description

Subfamily Eneopterinae Saussure, 1874
Tribe Lebinthini Robillard, 2004
Gnominthus Robillard & Vicente, gen. nov.
Type species. *Gnominthus baitabagus* Vicente & Robillard, sp. nov.

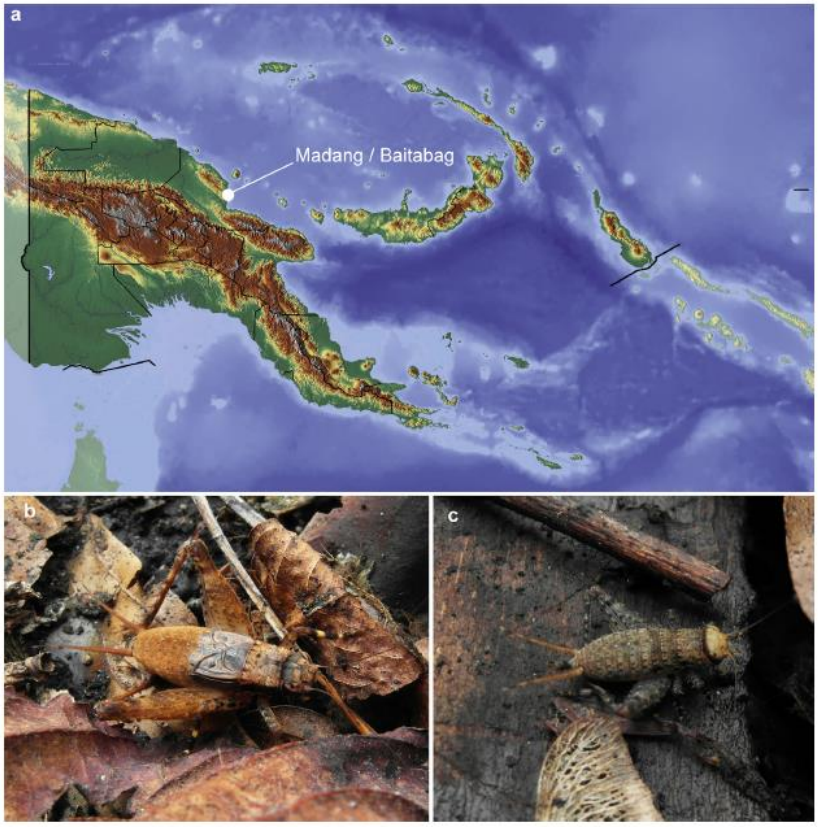

Fig. 1. Map of Papua New Guinea indicating the type locality of *Gnomtnthus baitabagus* (a); male habitus in leaf litter (b); juvenile on leaf litter (c).

Etymology. Genus named after the word "Gnome", the diminutive hairy mythical forest creatures of folklore, to refer to the stocky shape and hairy fastigium of these forest-dwelling crickets.

Diagnosis. Among Eneopterinae and Lebinthini genera, *Gnominthus* has a small size and a stocky shape. Genus characterized by the shape of head, with a very wide fastigium; eyes large compared to *Agnotecous* and *Centuriarus*, occupying almost the whole vertex length, but little prominent compared to *Lebinthus* and *Cardiodactylus* species; eyes higher than wide in lateral view; face higher than wide. FIII short and muscular. Microptery in both sexes, FWs not reaching mid- length of abdomen. **Male.** Dorsal and lateral fields of FWs almost of similar sizes, dorsal field slightly longer. FW venation characterized by large harp with a bisinuate oblique vein delimiting anteriorly a circular bump (tumescence) in middle of harp; diagonal vein curved posteriorly; 1A (file) vein bisinuate, as in *Cardiodactylus* and *Centuriarus*; mirror slightly differentiated, but small and not rounded; apical field small (one cell in E alignment); Sc vein with 1–2 bifurcations near apex. Male genitalia triangular, characterized by absence of individualised apical lophi; pseudepiphallus ended posteriorly by a short rounded plate, slightly concave dorsally; anterior apex raised, slightly indented; rami twice shorter than rest of pseudepiphallus; pseudepiphallic parameres forming a rounded plate ventrally. Ectophallic fold rounded apically, with two parallel sclerites. Endophallic sclerite very long, extended anteriorly as in *Lebinthus*, with lateral lamellas and a narrow dorsal crest. **Female.** FWs slightly overlapping, with an apical whitish spot on lateral edge of dorsal field. Ovipositor shorter than FIII, its apex slightly denticulate on dorsal edge. Female copulatory papilla rounded; its basal sclerite with a median expansion; apex rounded, folded ventrally.

Description

Size small, shape stocky. Head shape (Fig. 3a–c) differing from other genera of the tribe; fastigium wider than long (Fig. 3a), thrice as wide as scapes and prolonging the vertex without discontinuity. Eyes large but little prominent, higher than long, longer than wide; in dorsal view, eyes occupy most of vertex length and their combined width represents about 40% of head width (50% in *Lebinthus santoensis* and 46% in *L. bitaeniatus*) (Fig. 3a); head rather triangular in facial view, thinner than in *Lebinthus* species (Fig. 3c). Ocelli small and rounded, forming a wide triangle on fastigium. Pronotum dorsal disc almost rectangular, wider than long, its posterior margin

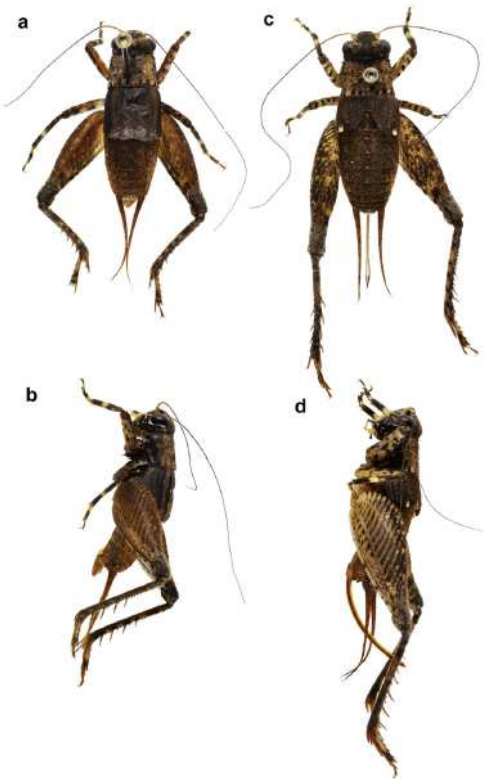

Fig. 2. General views *Gnominthus battabagus* in dorsal (a,c) and lateral (b,d) views: male (a,b); female (c,d). Scale bar, 1 cm.

straight (Fig. 2a–d). TI with 2 tympana, inner one (anterior) covered by a sclerotized expansion, its membrane visible along a small longitudinal slit only; outer tympanum (posterior) ellipsoidal, smooth. TI with 2 inner and 1 outer apical spurs. TII with 2 inner and 2 outer spurs. FIII short and muscular, without linear region before knee. TIII serrulated on their whole length, slightly furrowed longitudinally on dorsal face, and with 4 pairs of subapical spurs and 3 pairs of apical spurs; inner spurs long and curved, outer spurs shorter and straight. Tarsomeres III-1 with 2 dorso- apical spines and a row of spines on outer dorsal edge; without lateral outer spine. FWs short, not reaching abdomen mid-length; hindwings absent. Microptery in both sexes, FWs not reaching mid-length of abdomen.

Male. Metanotal glands absent. Dorsal and lateral fields of FWs almost of similar sizes, dorsal field slightly longer. FW venation (Figs 3e and 4): 1A vein (file) slightly bisinuate at level of angle, its transverse region located near FW third; stridulatory file with teeth on the transverse parts of 1A and few on its angle (Fig. 5a). Harp large, with one main oblique vein, bisinuate and delimiting anteriorly a circular bump in middle of harp; anterior region of harp with 3–4 additional faint parallel oblique veins. Diagonal vein slightly curved posteriorly. CuP absent, but claval fold visible along base of CuA in harp. CuA faint, slightly curved inward near apex. Median fold small, rectangular, located on dorsum as in *Lebinthus* and *Agnotecous*. Region posterior to harp with faint longitudinal veins, except for their apex, strong. Mirror slightly wider than other cells of D alignment, but little differentiated, not rounded. Cell c1 with a median transverse vein. Chord cells well differentiated. Apical field including only a few cells posterior to the mirror (alignment E). M strong and convex, orange, fused to R near FW apex, but very thin near fusion. Subgenital plate clog-shaped, twice as long as sternites; its inner side with baso-lateral swellings.

Male genitalia (Fig. 6a–d). Pseudepiphallus long and triangular, its basal margin slightly raised and indented, its posterior apex without paired lophi; ventral plate small, forming an incomplete circle similar to a horseshoe. Rami straight, short and parallel. Ectophallic apodemes divergent and long, widened apically. Ectophallic arc well sclerotized, straight, with a short anterior expansion. Ectophallic fold with 2 wide parallel sclerites forming an incomplete X shape; its apex triangular and membranous. Lateral ectophallic expansions forming wide plates basally, then membranous valves covering ventrally base of ectophallic fold. Endophallic sclerite very long, Y-shaped, extended anteriorly as in *Lebinthus*; endophallic apodeme with lateral lamellas and a narrow dorsal crest.

Female. FWs as long as in males (Figs. 2 a–d and 3 d), slightly overlapping, with an apical whitish spot on lateral edge of dorsal field. Dorsal field with strong longitudinal veins and faint transverse ones (Fig. 3d); lateral field with 4 longitudinal veins; Sc without bifurcations. Posterior margin of lateral field straight. Ovipositor laterally flattened, apex lanceolate and slightly denticulate dorsally (Fig. 7b).

Female genitalia. (Fig. 6a and b) Female copulatory papilla little sclerotized, rounded; basal sclerite forming a ring with a median dorsal expansion; apex rounded, folded ventrally.

3.2. Species description

Gnominthusbaitabagus Vicente & Robillard, sp. nov.

Type material. Male holotype. Papua New Guinea, Madang, forêt de Baitabag [forest of Baitabag], 5°08′20.8″S 145°46′26.4″E, 86 m, 6.XII.2012, nuit [night], litière [leaf litter], molecular sample LpBai[L38GnoBai] (TR2469) (MNHN-EO- ENSIF3553). **Allotype female**, same information as HT, adulte en élevage [adult in captivity] (MNHN-EO-ENSIF3554). **Paratypes** (13♂, 8♀): same locality as HT, adultes en élevage [adult in captivity]: 2♂ (F0-♂1-2), call recording (MNHN-EO-ENSIF3556-3557); 4♂ (MNHN-EO-ENSIF3558-3561); 2♀ (MNHN-EO-ENSIF616 -617); reared specimens F1: 5♂, call recording (F1-♂1-5) (MNHN-EO-ENSIF611-614, MNHN- EO-ENSIF43); 1♂, mating sequence video (F1-♂6) (MNHN-EO-ENSIF39); 3♂ (MNHN-EO- ENSIF40-42); 6♀ (MNHN-EO-ENSIF30-35).

Other material examined. Same information as HT, 1 juvenile, litière [leaf litter] (TR2470), molecular sample L108GnoBai2 (MNHN-EO- ENSIF3555); reared specimens F2: 1♂, call recording (F2-♂1) (MNHN-EO-ENSIF36); 1♂, 4♀ (MNHN); reared specimens F3: 6♂, 5♀.

Type locality. Papua New Guinea, Madang, forest of Baitabag, 5°08′20.8″S 145°46′26.4″E.

Distribution. Papua New Guinea, New Guinea Island, near Madang (Fig. 1 a).

Etymology. Species named after the type locality.

Diagnosis. *G. baitabagus* is characterized by its small size and stocky shape, contrasted coloration mixing orange brown, yellow and black. Characteristic shape of head, male venation with a circular bump in middle of harp, and genitalic characters are considered as diagnostic characters of the genus.

Description. Size small. Coloration constrasted, yellow, orange brown and black. Head dorsum (Fig. 3a) black with a Y whitish pattern between eyes, with a thin yellow band posterior to eyes; fastigium whitish or yellow, densely setose, with a black area pos-

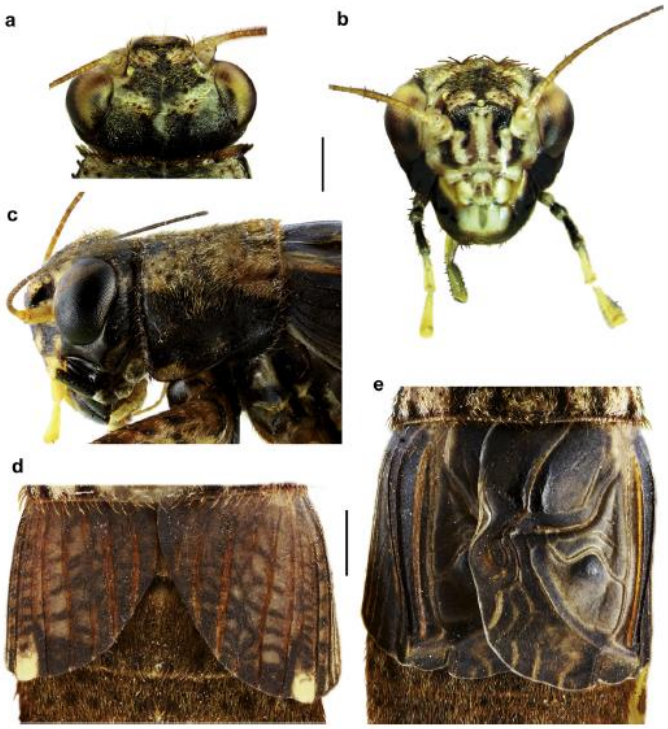

Fig. 3. Details of *Gnominthus battabagus* morphology: head of in dorsal (a), facial (b) and lateral (c) views; detail of female (d) and male (e) FW venation. Scale bars, 5 mm.

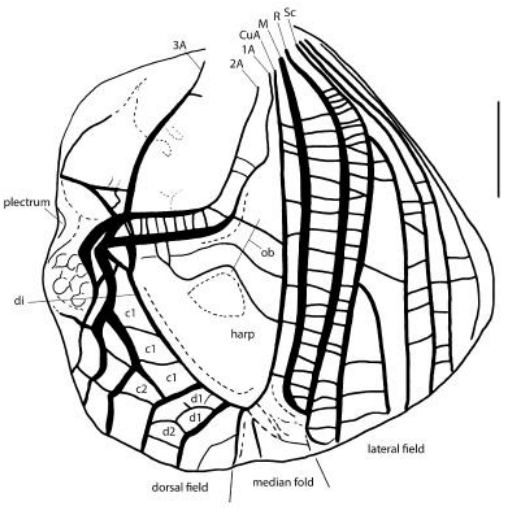

Fig. 4. Male forewing venation of *Gnominthus battabagus*. Dotted lines reprensent folds or three-dimensional patterns in the cells. Labels: see Section 2. Scale bar, 1 mm.

terior to median ocellus (looking like a heart-shape pattern in some specimens). Occelli yellow. Eyes black with a longitudinal orange brown band dorsally. Scapes small, whitish to light yellow with orange spots; base of antennae light yellow, then orange. Face with a triangular mask (Fig. 3b), whitish to yellow, from bellow eyes to clypeus, and including labrum and bases of mandibles; mask with more or less marked dark patterns including two black stripes bellow antennae and symmetrical curved patterns on front part of fastigium, and 2 small dark spots below median ocellus; rest of face and lateral part of head mostly black (Fig. 3b and c). Mandibles dark, except for the yellow margin near labrum; maxillary palpi black basally, their articles 4 and 5 yellow. Pronotum: Dorsal disk almost rectangular, wider than long, densely setose, its posterior margin straight, mostly light colored, yellowish to cream, with variable dark spots and patterns; posterior margin straight, completely dark in some specimens, yellow except for spaced dark spots in others. Lateral lobes black, with an irregular yellow pattern on ventral margin (Fig. 3c). Legs: Fore and mid legs with coxae and femur yellow with black spots. Trochanter yellow; TI-II black with a wide yellow ring (in some specimens an additional light ring near knee). For each pair of legs, tarsomeres 1 and 3 yellow basally, then black. FIII brown with strong striated dark brown patterns on outer faces and dark spots on ventral edges, knees dark brown; TIII dark brown with faint yellowish rings. Abdomen orange brown. Cerci mostly yellow brown with few faint dark rings near apex.

Male. FWs dark brown with orange veins, the median bump in middle of harp black. Vein 1A with 115 ± 2 (n=4) stridulatory teeth, most of them located on the transverse part of the file (102 ± 1),

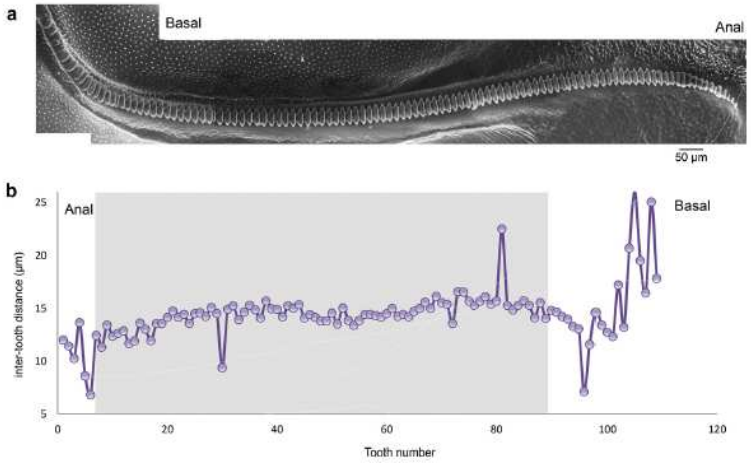

Fig. 5. Stridulatory file of *Gnominthus battabagus*. Photographs of the stridulatory file (right wing) in ventral view (a); file inter-tooth spacing measured in one male; the highlighted area indicates the file region used for sound production (b).

with a few teeth on the angle (13 ± 3) (Fig. 5a). Lateral field mostly black, including Sc and 3-4 strong ventral longitudinal veins; Sc black, with 1-2 bifurcations near apex; R black, strong along its whole length.

Male genitalia. Pseudepiphallus ended posteriorly by a short rounded plate little sclerotized, concave dorsally and slightly setose dorsally and ventrally, showing a trace of median suture. Pseudepiphallic parameres with a ventral lobe forming a rounded plate and a dorso-lateral lobe with sharp edges. Endophallic sclerite with short lateral arms and a short median, rounded posterior expansion; sclerite apex reaching basal margin of pseudepiphallus.

Female. FWs dorsal field dark brown with orange longitudinal veins (Fig. 3d). Posterior corner between dorsal and lateral fields forming an acute angle. Ovipositor rather short, as long as FIII.

Juvenile. Most instars brown mottled with black, face and anterior half of head dorsum whitish (Fig. 1c).

Measurements. see Table 1.

Habitat and life history traits. *G. baitabagus* is active at night in the leaf litter of forested areas.

4. Bioacoustical results

4.1. Description of the calling song

The calling song of *G. baitabagus* (Fig. 7) was recorded in the laboratory (24.5 °C, F1-male2, MNHN-EO-ENSIF612). Its echemes consist of short trills comprising 50 ± 8 syllables (n = 10 echemes). Each echeme has a rectangular amplitude profile and lasts for 0.9 ± 0.1 s, with a period of 7.5 ± 0.4 s. Syllables last 9.2 ± 0.8 ms, with a period of 17.1 ± 1.9 ms. The spectrum (Fig. 7g) shows a clear dominant peak at 21.7 ± 0.43 kHz corresponding to the third peak of an harmonic series (peak #1 ca. 7.2 kHz; peak #2 ca. 14.4 kHz).

4.2. Biomechanics of the calling song

The stridulatory file of *G. baitabagus* has a simple structure comprising ca. 115 simple teeth among which ca. 80 are likely to be functional according to their distribution pattern (Fig. 5). In the functional part (ca. 1.215 mm), the mean inter-tooth distance is 14.46 µm (tooth density = 69 teeth/mm). Given the rather homogeneous amplitude profile of the syllable, we assume that the closing movement of the forewings is more or less regular. Based on the measured syllable duration, the dominant frequency and inter-tooth distances, we can predict which frequency peak is generated by the tooth impacts.

Under the hypothesis H0 that stridulation generates the dominant peak at high frequency, the instantaneous velocity of the system would be very high (ca. 313 mm/s) and would involve ca. 190 functional teeth. This is far above the total number of teeth of the file, which invalidates this hypothesis.

The alternative hypothesis H1 is that stridulation generates the lower peak of the spectrum (ca. 7.2 kHz) and that the dominant, high-frequency peak, corresponds to the third harmonic of the low fundamental frequency, amplified by the resonance of the wing. Under this hypothesis, 80 functional teeth are sufficient to generate this low-frequency peak, and the corresponding instantaneous velocity of the system is ca. 104 mm/s (721,000 teeth/s * 14.46 µm).

The global estimated speed from syllable duration is 130.76 mm/s, which matches H1 and thus supports the hypothesis that the fundamental frequency is the low-frequency peak of the spectrum, i.e., that the tooth impact generates the low frequency and not the dominant peak at higher frequency. As a consequence, one tooth impact would generate three primary waves due to the vibratory properties of the resonator.

5. Behavioral results

The behavioral sequences observed in *G. baitabagus* are conform to usual cricket mating sequences: they starts with a courtship, which allows the subsequent production, transfer and retract of one spermatophore, followed by the consumption of this spermatophore by the female. Only one of four observed sequences included two successive behavioral cycles, with the production of two spermatophores of the same size. Males begin the courtship with antennal contacts to the female body, followed by singing and tapping with palpi, looking toward the female, while producing the spermatophore. When the spermatophore is ready for fecundation, the male starts singing and tapping with palpi almost continuously.

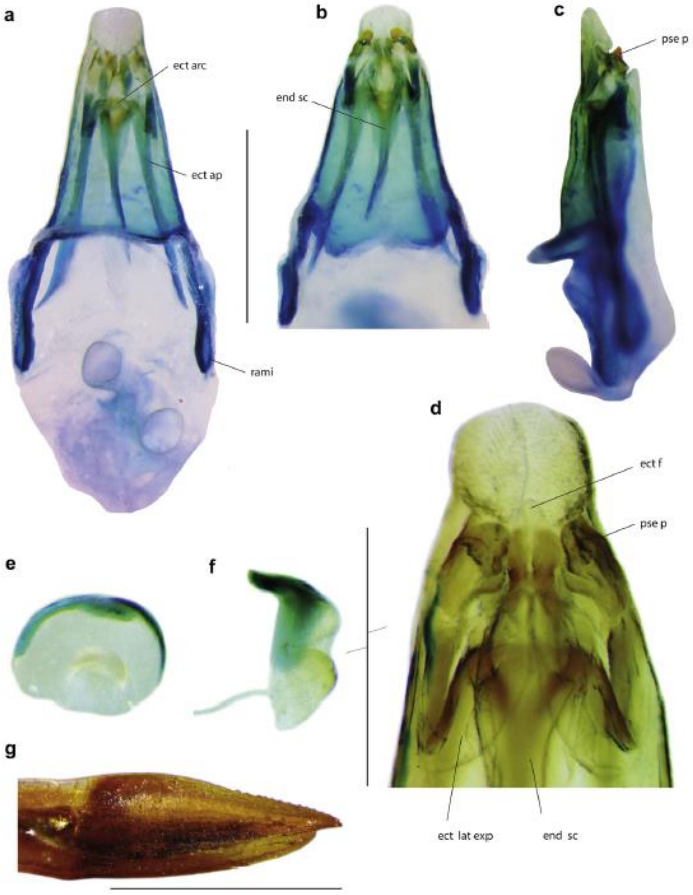

Fig. 6. Genitalia of *Gnominthus battabagus*: male genitalia in dorsal (a), ventral (b) and lateral (c) views, with a detailed view of posterior apex in ventral view (d); female copulatory papilla in ventral (e) and lateral (f) views; apex of ovipositor (g). Labels: see Section 2. Scale bars: d–f, 0.5 mm; a–c, g, 1 mm.

Table 1
Measurements of *Gnominthus battabagus* n. sp. Abbreviations: see Section 2.

	PronL	PronW	FWL	FWW	HWT	FIIIL	FIIIW	TIIIL
Male holotype	2.5	2.9	4.6	3.0	–	11.3	3.6	7.8
Males (n = 5)	2.4–2.7	2.9–3.6	3.9–4.6	2.6–3.0	–	10.0–11.6	3.5–3.9	7.3–8.1
(mean)	(2.57)	(3.24)	(4.32)	(2.86)	–	(10.92)	(3.7)	(7.72)
Female allotype	2.8	4.0	3.5	2.2	–	11.6	4.0	8.6
Females (n = 5)	2.6–2.8	3.7–4.0	2.8–3.5	2.2–2.6	–	10.3–11.6	3.6–4.0	7.7–8.6
(mean)	(2.72)	(3.86)	(2.98)	(2.46)	–	(11.08)	(3.9)	(8.06)

	TIIIs				TaIIIs	ST (n = 4)	OL
	las	Ibs	Oas	Obs			
Male holotype	5	4	10	6	4	?	–
Males (n = 5)	5–6	3–4	9–10	5–6	4–5	112–118	–
(mean)	(5)	(4)	(10)	(6)	(4)	(115)	–
Female allotype	7	4	9	4	4	–	9
Females (n = 5)	5–7	3–5	7–10	4–5	4	–	8–9
(mean)	(6)	(4)	(8)	(5)	(4)	–	(8.42)

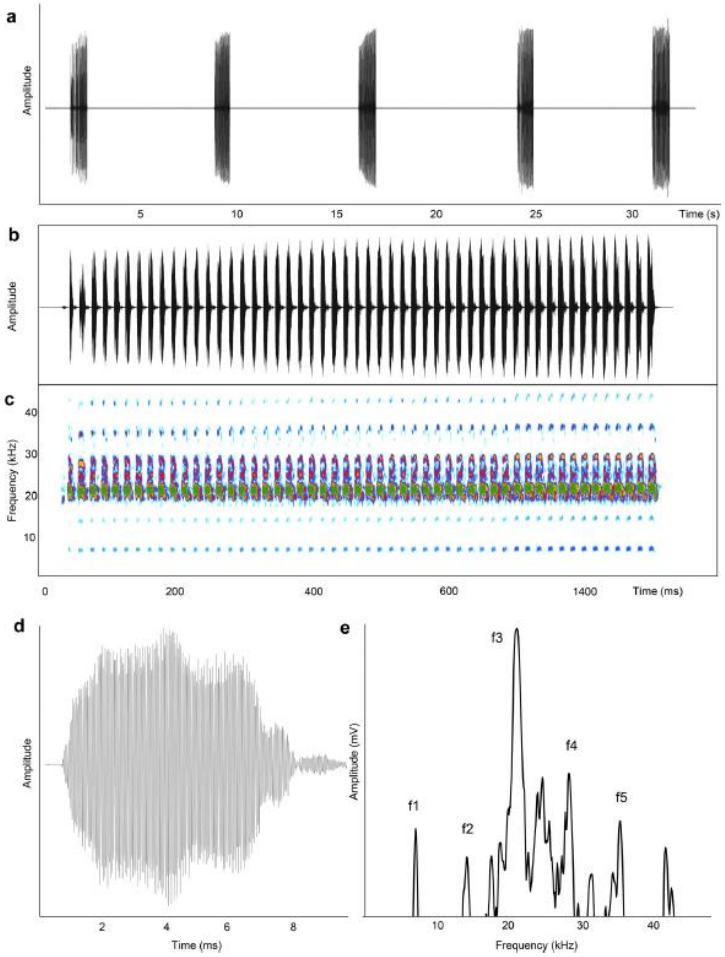

Fig. 7. Calling song of *Gnominthus battabagus*: oscillogram of 5 echemes (a); detailled oscillogram (b) and sonogram (c) of 1 echeme; detailed oscillogram (d) and frequency spectrum (e) of 1 syllable.

Mating starts with the male turning back and walking backward toward the female. Then, the copulation takes place, resulting in the transfer of the spermatophore, which takes between two and four seconds approximately; copulation ends with the male moving forward. The male then turns around, while staying before the female in a back-to-face position and keeps a guarding behavior. When the female tries to remove the spermatophore, the male instantly performs a jumping back, moving toward the female. In the observed matings, males perform the jumping back behavior between one and ten times per mating, until the females retracts and consumes the spermatophore. In the sequence involving two successive matings, a new spermatophore production begins after removal of the previous one by the female, then the male turns around, antennates and sings. The female retracts the spermatophore when the production of a new one has already started, which means that there is a slight overlap between the guarding and the time of production of a new spermatophore. This second cycle continues in the same way as the first, until the couple separates.

6. Discussion

6.1. Diversity and endemism of Lebinthini in New Guinea

With *Centuriarus* (Robillard, 2011a), the new genus *Gnominthus* is the second endemic genus recently described from the eastern part of New Guinea Island, while the genus *Cardiodactylus*,

distributed widely in South-East Asia and in the Western Pacific region, presents several endemic species in this area (Otte, 2007; Bhowhmik, (1979) 1981). Thus, as new data accumulate, it becomes obvious that the diversity of crickets has been largely underestimated in New Guinea. Not mentioning rare species or species living in very specialized habitats, the fact that common and abundant species found in usual habitats are still unknown is the mark that barely any study has ever been performed in a given place. It is the case for the forest of Baitabag (Baitabag mission), which has been a classical field station near the city of Madang for many decades for other groups of animals including insects (Sirivanakarn, 1968; Lucky and Ward, 2010). The fact that abundant species present in the leaf litter such as *G. baitabagus* could be collected for the first time in 2012, with no previously collected specimen in any of the numerous insect collections examined (TR pers. observ., see list in Robillard et al., 2014) is an indication that a large amount of orthopteran diversity probably yet has to be discovered. Regarding eneopterine crickets, the presence of endemic genera and species with diversified shapes, bioacoustics and behaviors, such as described here, also suggests that New Guinea occupied an important place in the diversification of these crickets.

6.2. Acoustic diversity

When high-frequency songs were first detected in the eneopterines, two contrasted kinds of frequency spectra were recognized. They were considered either as convergences or as successive evolutionary steps in the use of harmonics as dominant frequencies (Robillard and Desutter-Grandcolas, 2004a; Robillard et al., 2007): in all the species of *Cardiodactylus* documented so far, the dominant peak corresponds to the third peak of the spectrum (Robillard et al., 2007, 2014; Robillard and Ichikawa, 2009; Robillard, 2009, 2011; Tan and Robillard, 2014). In contrast, in most of the species reported from the brachypterous clade of Lebinthini, the dominant frequency is the second peak of the spectrum (Robillard et al., 2007, 2010; Robillard, 2009). This picture is however questioned by several recent studies which suggested that this is perhaps a simplification of a larger spectral diversity. First, the biomechanical study of *Agnotecous robustus* showed that the dominant frequency of this species is in fact not a harmonic of the first peak, but a high fundamental frequency generated by an accelerated tooth impact rate (Robillard et al., 2013). Second, a few *Lebinthus* species presented songs that did not possess the same frequency spectrum as for the species previously described. For example, the species *Lebinthus villemantae* showed high dominant frequencies (ca. 20 kHz) corresponding to the first peak of the spectrum (Robillard, 2010). Also, the recent study of the Neoptropical genera *Ligypterus* and *Ponca* (Robillard et al., 2015) suggested that theses lebinthine's songs possess a third-harmonic dominant frequency, which is not directly produced by the tooth-plectrum impacts (i.e., f3 is a real harmonic of a lower fundamental frequency).

The new genus and new species presented here introduce a brand new pattern in terms of song spectrum to the brachypterous lebinthines: *Gnominthus* is indeed the first genus which clearly belongs within the paraphyletic group currently known as *Lebinthus* and that does not present a spectrum with a "second-peak" dominant frequency (revision in preparation, relationships based on both morphological observations and unpublished molecular phylogenetic results). Instead, *G. baitabagus* shows a spectrum with a third-peak dominant frequency, similar to that of *Cardiodactylus* species. The genera *Ligypterus* and *Ponca*, which also present this spectral pattern, are indeed at best the sister group of all other Lebinthini (Nattier et al., 2011), and their song spectrum could thus be explained by the fact that they share a similar kind of frequency filter with *Cardiodactylus*. Things are different for *G. baitabagus*, which is nested within the clade (*Lebinthus*-*Agnoteocous*), which species are generally characterized by a plectrum with a "second-peak" dominant frequency. It suggests either multiple origins of the second-peak spectrum and/or of the third- peak one, or a more complex history, depending on the phylogeny of the tribe. In any case, the whole lebinthine clade is probably more diversified than expected, in terms of spectral patterns, but also regarding the mechanisms responsible for these patterns.

6.3. Diversity of mating behavior

Cricket calling songs are characterized by their diversity and species-specificity (Alexander, 1962), but they are only the first step of a series of filters playing a role in the reproductive isolation of the species. Each species is then more or less characterized by a specific calling song, but also by diverse sequences of courtship and copulatory behaviors (Alexander and Otte, 1967). However, if acoustic data are now more or less common in studies describing new crickets, data about mating sequences are at best very rare.

In this paper, we present a preliminary study of the mating behavior of the new species *G. baitabagus*. The results suggest rich and diverse interactions between male and female as part of mating sequences. In previous papers about other eneopterines, it has been shown that these cricket's mating sequences possess the multiple copulations and that they can use varied communication channels during guarding after spermatophore transfer, including tremulations (vibrations), more or less intense acoustic activity, or even visual cues (Preston-Mafham, 2000; Narvaez and Robillard, 2012). In *G. baitabagus*, the observed couples present little or no tendency for multiple matings: out of four couples analyzed here, only one included two spermatophores in the same sequence. This tendency may be due to the low number of observations, or it can be an indication that multiple matings are not as generalized among these crickets as hypothesized before, and that alternative mating strategies might exist.

The mating sequence itself is conforming with other cricket species. It involves little singing activity, palpi interactions and antennations and also more uncommon acts. Clearly, the most interesting behavior in this species occurs during the guarding time: when the female tries to remove the spermatophore, while the male is almost turning his back, the male instantly performs a violent jumping back, moving toward the female. In the observed matings, males performed this particular behavior between one and ten times per mating, until the females retracts and consumes the spermatophore. This behavior seems to be initiated by the female's attempt to remove the spermatophore and its consequence seems to stop the female. It possibly avoids the premature withdrawal of the spermatophore by the female and would thus allow a longer fertilization process. The fact that the male is almost turning his back after mating, while apparently actively guarding the female, is another particularity of the species. It may indicate that the male's attention is not entirely on the female during this step, but that it is partly oriented toward detection of other kinds of stimuli, such as, maybe, presence of predators or male competitors. These first observations will need to be completed by precise quantitative analyses based on more observations and their interpretations will have to be tested using experimental protocols, in particular to investigate the roles of the new guarding behavior and to compare with other eneopterine species.

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Further reading

Robillard, T., 2011b. New *Cardiodactylus* species from unsuspected places in Southeast Asia (Orthoptera, Grylloidea, Eneopterinae). Zootaxa 2909, 14–26.

GENERAL CONCLUSIONS

In this thesis we outlined the main aspects regarding the biogeography, the bioacoustics and taxonomy of Neotropical crickets Eneopterinae. The world-wide dated biogeographical model for Eneopterinae fits well with general predictions proposed for the evolution of taxa with disjunct distribution and gondwanan origin (Sanmartin & Ronquist, 2004). Our result shows two colonizations of South America with ancestral using a boreotropical route of migration for the second colonization. We also show the most plausible scenario of high-frequency songs evolution in Eneopterinae, with dates and centers of origin. These results indicate two independent origins: one in South America (during early Eocene) and a second in Southeast Asia (during late Paleocene and early Eocene). Our study also explores the diversity in *Ligypterus* an Eneopterinae genus encompassing cryptic species. We demonstrate that the 5 described species of *Ligypterus* may represent just a fraction of the total fauna, with estimates of twice this number for the real diversity. We believe that the perspectives now open by discussion on this thesis have the potential to offer base for further studies regarding this subfamily and crickets in general. The exploration of the use high frequency communication in other cricket taxa is urgent, given the importance of this feature in the evolution and ecological processes in Eneopterinae. The use of DNA based method may clarify other taxonomic complex crickets taxa, unveiling the real diversity in Orthoptera.

Apendix I – Robillard et al. 2015 - Bioacoustics

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Bioacoustics of the Neotropical Eneopterinae (Orthoptera, Grylloidea, Gryllidae)

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In members of the cricket subfamily Eneopterinae (Orthoptera, Grylloidea), songs with powerful high-frequency (HF) harmonics have evolved, which likely represents a distinctive acoustic adaptation. In this study, we analysed or reanalysed the songs of the three eneopterine genera present in the Neotropics to evaluate whether they also possess high-amplitude HF components. We present new data and combine several lines of evidence to interpret or reinterpret the calling signals of a representative species for each genus. We used new recordings in order to detect and analyse potential HF components of the songs. Stridulatory files were measured, and stridulation was studied using high-speed video recordings. The results suggest that all eneopterine genera from the Neotropics use HFs to communicate, based on the rich harmonic content of their songs. Strikingly, the Neotropical eneopterines possess high dominant frequencies, recalling the patterns observed in the tribe Lebinthini, the most speciose tribe of the subfamily distributed in the Western Pacific region and in Southeast Asia: *Ligypterus* and *Ponca* show dominant harmonic peaks, whereas *Eneoptera* possesses unique features. The three species under study, however, deal differently with HFs.

Keywords: cricket; calling song; dominant frequency; harmonics; high frequency

Introduction

Orthopteran insects of the suborder Ensifera (crickets, mole-crickets, katydids) typically stridulate by rubbing their forewings (FWs) against each other. The sound signals emitted by the males are used mainly to attract females, and are therefore a key trait for reproductive success and survival. During FW closure, the plectrum of the lower wing (the left one) hits a series of cuticular teeth under the upper wing (i.e. the stridulatory file, located under the right wing). The vibrations produced by tooth strikes are subsequently amplified by wing resonance, through the enhanced vibration of the wings' membranes. In crickets, each tooth strike generates a sinusoidal elementary oscillation of the wing resonator, which can be sustained in time if subsequent tooth strikes occur at the correct time and phase (Bennet-Clark and Bailey 2002; Bennet-Clark 2003; Montealegre-Z et al. 2009). The closing part of the song syllable thus corresponds to the sum of the elementary oscillations produced by all the teeth involved in a given plectrum-file sweep.

The carrier or dominant frequency (fd) of the sounds produced by this mechanism varies according to the morphological and behavioural features of the species. The carrier or fd refers to the frequency in the call that contains the most energy. In addition to the

temporal pattern of male calls (Pollack 2000), carrier frequency can transmit important information about the identity and biological state of the emitter, although much information relies on the temporal pattern (Bertram et al. 2011). High-frequency (HF) and ultrasonic calls are common in the tettigoniids (Orthoptera, Ensifera, Tettigonoidea) (e.g. Morris et al. 1989; Bailey 1991; Montealegre-Z and Morris 1999), some of which can produce sound frequencies >100 kHz (Montealegre-Z et al. 2006; Sarria-S et al. 2014). In contrast, signals with frequencies above 10 kHz have rarely been documented in crickets, which typically produce low-frequency (LF), pure-tone signals (2–8 kHz: e.g. Dumortier 1963; Leroy 1966; Robinson and Hall 2002; Montealegre-Z 2009). Recent studies, however, have demonstrated that members of at least one cricket subfamily, the Eneopterinae (Orthoptera, Grylloidea), have evolved songs with powerful HF harmonics. In the calls of the tribe Lebinthini, these frequencies became the fd, making some of these species the first crickets known to communicate with HF (>10 kHz), and in some species with ultrasonic (26–28 kHz) signals (Robillard and Desutter-Grandcolas 2004a; Robillard et al. 2007, 2013). This shift to HFs might represent new possibilities for diversification because HFs may not be perceived by other species, including potential predators or parasites.

In this study, we aimed to analyse the bioacoustics of the eneopterine species from the Neotropical region. Indeed, although Eneopterinae are distributed worldwide in tropical areas, they are only represented in the Neotropics by approximately a dozen species belonging to three genera (*Ponca*, *Ligypterus* and *Eneoptera*) (Robillard and Desutter-Grandcolas 2005, 2013). Although these species were first considered to belong to the same tribe Eneopterini based on few morphological evidences (Robillard and Desutter-Grandcolas 2004b), these genera likely have polyphyletic origins, as suggested by the more recent molecular phylogenetic studies (Robillard and Desutter-Grandcolas 2006; Nattier et al. 2011). According to these previous results, the clade (*Ligypterus*–*Ponca*) might be closely related to the tribe Lebinthini, the most speciose tribe of the subfamily that is distributed in the Western Pacific and Southeast Asia, while the genus *Eneoptera* would have an earlier origin within the subfamily. These relationships need, however, to be confirmed by further studies including additional molecular marker and in-depth taxonomical samplings of Neoptropical eneopterines, but bioacoustical evidence could be considered as well.

The bioacoustics of these species was not well studied in the past. No calling song descriptions have been published for any species in the genus *Ponca*, and although initial descriptions are available for the songs of two *Ligypterus* species (Robillard and Desutter-Grandcolas 2005) and two *Eneoptera* species (Desutter-Grandcolas 1998; Robillard and Desutter-Grandcolas 2005, 2011a, 2011b; Miyoshi et al. 2007), these recordings did not capture frequencies >10 kHz, a frequency range that we now know to contain significant energy in the songs of many eneopterine crickets. Despite the technical constraints of these earlier recordings, energy at HF harmonics can be detected in the calls of *Ligypterus*. The calls of the two *Eneoptera* species appear to be very different: *Eneoptera surinamensis* (De Geer, 1773) has typical stridulatory teeth and produces a LF continuous trill showing a slight amplitude modulation. *Eneoptera guyanensis*, on the other hand, produces a unique trilling song that alternates between a low and a high fd range. These two frequency ranges are produced by two different wing movements that use different parts of the stridulatory file (Robillard and Desutter-Grandcolas 2011a).

In this paper, we present new data and combine several lines of evidence to study the calling signals of a representative species for each Neotropical genus. New recordings made using a higher sampling rate and a microphone with a more uniform frequency

response allowed us to detect and analyse HF components of the songs. Stridulatory files were measured and stridulatory movements were studied using high-speed video (HSV) recordings. For each species, the parameters describing the stridulatory behaviour were estimated by integrating morphological and acoustic information.

Materials and methods

Taxonomic sampling and studied specimens

The Neoptropical eneopterines consist of three genera belonging to the tribe Eneopterini (Robillard and Desutter-Grandcolas 2004a, 2008; Eades et al. 2014). This tribe is most likely polyphyletic according to the most recent molecular phylogenetic studies (Robillard and Desutter-Grandcolas 2006; Nattier et al. 2011), which suggested that the clade (*Ligypterus*–*Ponca*) might be closely related to the tribe Lebinthini, while *Eneoptera* would be more distantly related, with an earlier origin within the eneopterines. We analysed calls from one species of each Neotropical genus: *Eneoptera guyanensis* (Chopard, 1931), *Ligypterus fuscus* (Chopard, 1920) and *Ponca venosa* (Hebard, 1928). Studied specimens came from field collections in French Guiana and Costa Rica: the studied male of *E. guyanensis* ($n = 1$) was collected in March 2013 by J. Orivel in Petit Saut, French Guiana (N05°03′55.9″ W53°02′53.3″); males of *L. fuscus* [$n = 2$; $n = 1$ for HSV recording] were collected in July 2011 by Laure Desutter-Grandcolas of the Muséum National d'Histoire Naturelle (MNHN) in the Station Biologique des Nouragues, French Guiana; and males of *P. venosa* ($n = 1$ for acoustic analysis; $n = 2$ for morphological study) were collected in March 2005 by Laure Desutter-Grandcolas (MNHN) in La Selva field station, Costa Rica.

Acoustic analysis

The basic cricket song terminology used here follows Ragge and Reynolds (1998). One song unit is called a syllable and corresponds to the sound produced by one opening–closure cycle of the male FWs. A group of syllables constitutes an echeme, which represents a call unit in terms of communication.

E. guyanensis, *L. fuscus* and *P. venosa* were recorded in the laboratory from specimens collected in the field as juveniles or as nymphs. Recordings of *L. fuscus* and *E. guyanensis* were made with a modified condenser microphone (CM16, Avisoft Bioacoustics, Berlin, Germany; frequency range: 3–150 kHz ± 6 dB, R. Specht, pers. comm.) in a room lined with sound attenuating foam and controlled for temperature. The insects were placed overnight in a net cage, to reduce echoes, with the microphone hanging 30 cm above the cage. Automatic recordings were made using the program Avisoft Triggering Harddisk Recorder version 2.97 and an 8-Pre MOTU sound card at a sampling frequency of 96 kilo-samples per second (16 bit). After checking for homogeneity among the recorded individuals, acoustic features were measured from sound recordings of one male of *Eneoptera* (song temporal pattern previously analysed in detail in Robillard and Desutter-Grandcolas 2011a) and two males of *Ligypterus* (Tables 1–4).

To correct for the frequency response of the microphone and generate audio files with accurate power spectra, we applied a user-defined frequency response filter under Avisoft-SAS-Lab Pro version 4.40 (finite impulse response (FIR) filter: Specht 2008). The FIR filter was generated using the inverse of the microphone frequency response provided by the manufacturer.

The male of *P. venosa* was recorded in a plastic box 10 × 5 × 5 cm, using a Sony TDC-D8 DAT Recorder with a Sony ECM-MS907 directional microphone placed at about

Table 1. Morphological, acoustical and mechanical measurements associated with stridulation.

	Total file length (mm)	No. of teeth (total)	No. of teeth (used)	Tooth density (no/mm)	Total distance used (mm)	Average tooth distance (μm)	TSR (strikes/s)	Syllable duration (ms)	Candidate fundamental frequency (kHz)	Global speed from syllable duration (mm/s)	Instantaneous speed from frequency (mm/s)
E. guyanensis ($n = 1$)											
Simple teeth – LF	1.20	58	14.7 ± 0.7	45	0.33	22.14	4083	3.6 ± 0.4	3.83 ± 0.04	92	85
Double teeth – HF	1.15	57	26.4 ± 3.8	54.6	0.48	18.3	9778	2.7 ± 0.5	19.99 ± 0.09	179	183
[crests]		[114]	[52.7 ± 7.6]	[109]		[9.17]	[19,518]				
L. fuscus ($n = 2$)	1.75 ± 0.02	98 ± 1.4	60	62.9 ± 3.8	0.96 ± 0.06	15.91 ± 0.95	7500	8 ± 0.6	**H0: 6.48 ± 0.06 (*)** H1: 19.73 ± 0.41	121	**H0: 103 (*)** H1: 314
P. venosa ($n = 2$)	2.88 ± 0.10	369 ± 14	266 ± 4	140 ± 10	1.91 ± 0.17	7.14 ± 0.55	2852	93.25	**H0: 5.98 (*)** H1: 18.045	20.5	**H0: 42 (*)** H1: 128
G. bimaculatus ($n = 6$)	4.7 ± 0.21	156	90	35.71	2.52	30.11 ± 1.18	4500	20	4.8	126	145

Notes: No. of teeth used is estimated from the pattern of teeth distribution in *L. fuscus* and *P. venosa*, and from syllable oscillograms only in *E. guyanensis*. The candidate fundamental frequency represents the different frequency peaks likely to correspond to the TSR; they are described as the null hypothesis (H0) for the lowest peak and H1 for the higher peak (harmonic peak); the preferred hypotheses according to the comparison between TSR, candidate frequency and instantaneous speed derived from peak frequency are represented in bold with a (*) symbol. For *E. guyanensis*, the morphological measurements given in brackets refer to the values taking the crests of the double teeth into account.

Table 2. Song parameters of *L. fuscus*.

		No. of syllables	Syllable duration (ms)	Syllable period (ms)	fd (kHz)	Freq. peak 1 (kHz)	Amplitude peak 1 (dB)	Freq. peak 2 (kHz)	Amplitude peak 2 (dB)	Freq. peak 3 (kHz)	Amplitude peak 3 (dB)
Male 1	Mean	30.75	7.12	16.29	19.41	6.37	−51.89	12.60	−53.13	19.40	−21.59
	SD	±3.23	±0.46	±0.28	±0.063	±0.05	±0.96	±0.30	±0.49	±0.05	±1.32
Male 2	Mean	33.48	8.86	12.81	20.06	6.59	–44.74	13.01	–31.35	20.06	–13.31
	SD	±2.38	±0.64	±0.47	±0.77	±0.06	±1.41	±0.093	±16.58	±0.77	±1.33
	Mean	32.11	8	14.55	19.73	6.48	− 48.32	12.81	− 42.24	19.73	− 17.45
	SD	±2.80	±0.6	±0.37	±0.42	±0.06	±1.18	±0.20	±8.53	±0.41	±1.32

Notes: The silent gaps at the beginning of the echeme were removed prior calculation of the syllable period.

Table 3. Example of echeme structure of *L. fuscus* (male 2).

Counts		Chirp 1		Chirp 2		Chirp 3		Chirp 4		Trill	Period (s)
Total no. of syllables	No. of chirps	No. of syllables	Inter-chirp period (ms)	No. of syllables	Period (ms)	No. of syllables	Period (ms)	No. of syllables	Period (ms)	#Syllables	Between echemes
30.75 ± 3.23	3 ± 0.65	2	177.81 ± 63.72	2.45 ± 0.51	94.58 ± 27.88	3.25 ± 0.68	60.95 ± 11.52	4.25 ± 1.89	56.33 ± 6.29	22.85 ± 3.30	4.92 ± 0.80

Note: Inter-chirp periods of 19 ms or greater were used to separate chirps.

Table 4. Song parameters of *P. venosa.*

	Syllable			Frequency peak 1		Frequency peak 2		Frequency peak 3		Frequency peak 4	
	Duration (ms)	Period (s)	fd (kHz)	(kHz)	Amplitude (dB)	(kHz)	Amplitude (dB)	(kHz)	Amplitude (dB)	(kHz)	Amplitude (dB)
Whole song	93.25	7.1	6.0	6.0	− 37.6	11.6	−53.8	18.0	− 41.5	–	–
	±3.85	±2.4	±0.1	± 0.1	± 1.0	±0.3	±1.5	± 0.4	± 1.1	–	–
First part of song	56.6	–	5.8	5.8	0.0	11.2	−15.6	16.8	−5.0	–	–
	±2.1	–	±0.4	±0.4	±0.0	±0.2	±2.8	±0.2	±2.9	–	–
Last part of song	39.3	–	6.2	6.2	0.0	11.9	−19.2	14.5	−21.4	18.34	−2.78
	±2.9	–	±0.0	±0.0	±0.0	±0.2	±2.5	±0.3	±3.3	±0.26	±2.61

10 cm from the box. This microphone has a relatively uniform frequency response between 100 Hz and 15 kHz, which is not sufficient to assess the HF harmonics of the song, but it provides an idea of the song features of this species. We recognize, however, that the frequency measurements for this species are limited to the audio range and that new recordings will need to be made with appropriate equipment to determine the upper frequency range of the species' song.

Temporal and spectral song features were measured using the automatic commands under Avisoft-SASLab Pro. We measured syllable duration, syllable period (time from the start of one syllable to the start of the next), fd (frequency with the most energy, kHz), and frequencies and relative amplitudes of each harmonic. All recorded files are deposited in the Sound Library of the Muséum National d'Histoire Naturelle, Paris.

Recordings of stridulatory movements

Stridulatory wing movements were recorded in the laboratory from one male of *L. fuscus* and one male of *E. guyanensis* using an HSV camera (AOS Technologies AG, Baden-Daettwil, Switzerland) allowing for the characterization of the wing motion of these species. Video recordings were obtained at 3000 frames/s, whereas sound was recorded simultaneously at a 96 kilo-samples per second sampling rate. Although we could not calculate the exact speed of stridulatory movement, these HSV recordings allow us to distinguish between the smooth wing closure typically seen in Gryllinae species (e.g. *Gryllus campestris*, *Gryllus bimaculatus* or *Teleogryllus oceanicus*) (Koch et al. 1988; Bennet-Clark 2003; Montealegre-Z 2005), a discontinuous wing closure incorporating silent intervals, as has been documented in some eneopterine species (Robillard et al. 2013), or a more complex closing–opening pattern as suggested in *E. guyanensis* (Robillard and Desutter-Grandcolas 2011a), but which requires formal confirmation.

Morphology

To characterize the morphology of the stridulatory teeth, files of recorded males were photographed using an AmScope MU1000 digital camera (http://www.amscope.com) at a magnification of 115 ×. Stridulatory file measurements were made using the dimension tool of the program ImageJ (Schneider et al. 2012) from digitized images assembled using the software Microsoft ICE.

Stridulation analysis

To determine whether the fd of the song corresponds to the natural fundamental frequency or to a higher harmonic, we compared wing velocity measurements, calculated as the length of functional region of the file divided by the whole FW closing time (= syllable duration), with instantaneous song frequency, calculated by dividing the average inter-tooth distance within the functional region by the time separating two subsequent waveforms in the song, under a candidate value of fundamental frequency ($1/f$). The instantaneous song frequency that most closely matches the wing velocity measurement is then supposed to correspond to the tooth impact rate (hereafter tooth-strike rate, TSR). Irrespective of the relative energy of each peak of the spectrum, if the first peak matches the tooth impact, it means that each functional file tooth produces one song elementary waveform and that fd is directly produced by the stridulatory mechanism, as described for Gryllinae (e.g. Montealegre-Z et al. 2009). However, if the estimated wing velocity

matches a higher instantaneous frequency, it suggests that more than one waveform is produced per tooth strike, because of the harmonic vibration of the wings, as suggested for some other eneopterine crickets (Robillard et al. 2007, 2013).

As a corollary, under the null hypothesis that TSR produces fd, the time between two successive tooth strikes (1/fd), multiplied by the number of teeth available for stridulation (the relatively straight transverse part of the file) should approximate the measured syllable duration. Similarly, under this hypothesis, the number of elementary waves in the syllable (excluding the free vibration at the end) should more or less match the number of teeth available in the rectilinear part of the stridulatory file.

Results

E. guyanensis

The stridulatory file of *E. guyanensis* has been described previously (Desutter-Grandcolas 1998; Robillard and Desutter-Grandcolas 2011a) and was not reanalysed here. Briefly, it consists of two distinct regions believed to be involved in the two distinct parts of the song: the basal part of the file is made up of large teeth that look like normal cricket teeth, whereas the distal region is made up of double teeth, each one being subdivided by a median furrow into two parallel crests, resulting in more or less doubling the density of functional teeth in this region. The mean inter-tooth distance is 22.14 μm in the simple teeth area (tooth density = 45/mm), the distance between the crests of the double teeth in the double-teeth region of the file is 8.42 μm (crest density = 119/mm).

The song of *E. guyanensis* (Figure 1), analysed with new sound recordings and HSV, conforms in temporal pattern to previous descriptions: it is a continuous trill composed of HF and LF sections (see Robillard and Desutter-Grandcolas 2011a for measurements of temporal parameters). The HSV confirms that the sound pulses in the song are true syllables generated during a single cycle of FW opening–closing. HSV also shows that there are two types of FW closing–opening cycles, as hypothesized previously from lower speed video: the HF syllable is generated using the distal region of the file made of double teeth, and the LF syllable is generated using the basal region of the file made up of simple teeth. Both types of syllables correspond to a rather low-amplitude movement (Figure 2, see Supplementary Video 1).

The most surprising results are the spectral components of both parts of the song (Figure 1). For the HF part, the fd was measured as 19.96 ± 0.09 kHz instead of 14.61 ± 0.55 kHz, as previously measured, which represents a difference of 5.5 kHz. There is a very low-amplitude harmonic at ca. 40 kHz suggesting that the 20 kHz peak constitutes a fundamental frequency ($f1'$) generated by the crests of the double teeth.

For the LF part of the song, the new recordings confirm the previously measured low fundamental frequency ($f1$ = 3.83 ± 0.04 kHz); however, here again the HF content of the spectrum could not be estimated in previous analyses. The spectrum shows five harmonic peaks including the fundamental ($f1$), and the fifth harmonic ($f5$ = ca. 20 kHz) is either co-dominating or sometimes even dominating over $f1$. These discrepancies with the previous analyses of the song of *E. guyanensis* are obviously related to the rather LF response of the previously used microphone, which greatly under-sampled frequencies above 10 kHz.

Based on these new data, we estimate that the LF syllables are produced using 14–15 teeth at a speed of ca. 90 mm/s, and that the HF syllables are produced using ca. 26 double teeth (or 52 crests), hit by the plectrum at a speed of ca. 180 mm/s.

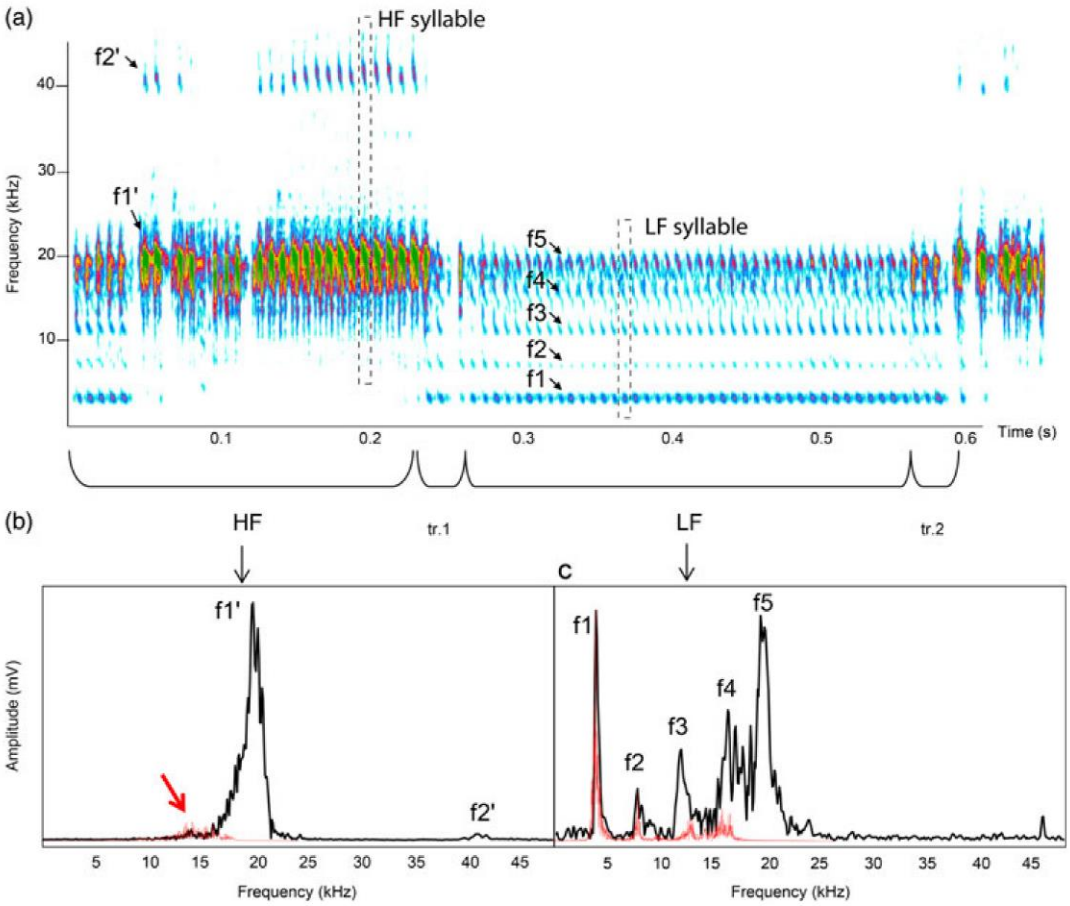

Figure 1. (Colour online) Song of *E. guyanensis*. Sonogram of a bout of HF and a bout of LF song including transitions (tr.1 and tr.2) (a). Frequency spectrum of one HF syllable (b) and one LF syllable (c). The red spectrum corresponds to the songs as analysed in previous papers (Desutter-Grandcolas 1998; Robillard and Desutter-Grandcolas 2011a). Arrows point to the fundamental frequencies $f1$ and $f1'$ with their respective harmonics ($f2$, $f3$, $f4$ for fundamental $f1$; $f2'$ for fundamental $f1'$).

L. fuscus

The file of *L. fuscus* has a simple structure compared with *E. guyanensis* (Figure 3(a)). It comprises ca. 100 simple teeth among which 60 are likely to be functional. In the functional part, the mean inter-tooth distance is 15.91 ± 0.95 µm (tooth density = 62.9 ± 3.8/mm).

As described in Robillard and Desutter-Grandcolas (2005), *L. fuscus* has a short, irregular song made up of 32 ± 2 syllables (Figure 4; Tables 2 and 3). It typically begins with one to four chirps consisting of two to four syllables each and ends with a short trill (see Table 3 for the detailed analysis of a male's song structure). The syllable duration is almost constant (8 ± 0.6 ms), having a mean period of 14.5 ± 0.3 ms (after removal of the gaps between the initial groups of syllables). The song spectrum consists of a harmonic series based on a fundamental frequency of 6.48 ± 0.05 kHz. However, contrary to what was found previously based on LF recordings, the LF peak of 6.48 kHz is not the fd and it is even more than 30 dB below the true dominant peak frequency of 19.73 ± 0.41 kHz, which corresponds to the third peak of the spectrum.

HSV shows that wing motion is regular (Figure 3(b); see Supplementary Video 2) during FW closing phase. It means that stridulation speed estimated from syllable duration

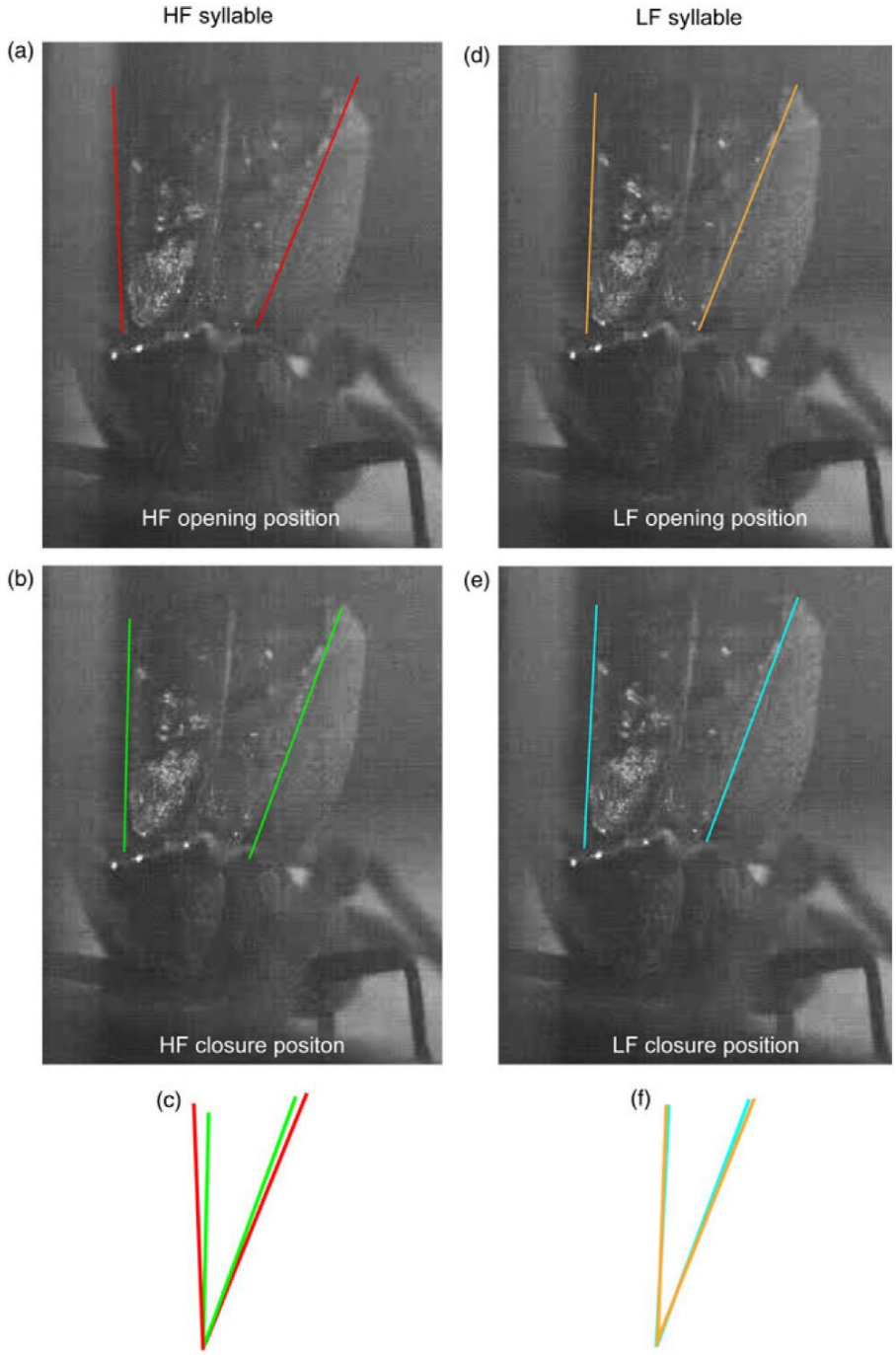

Figure 2. (Colour online) Snapshots from HSV recording of a singing male *E. guyanensis* showing the different positions of the FWs during the two types of closing–opening cycles, corresponding to the HF syllables (a–c) and to the LF syllables (d–f). Colour lines have been placed on the photographs for each position to represent the external limits of the FWs. For each type of syllable, the colour lines have been superimposed in the lower panels (c and f) to visualize the respective amplitude of the closing movement. The stridulatory movement is shown in Supplementary Video 1.

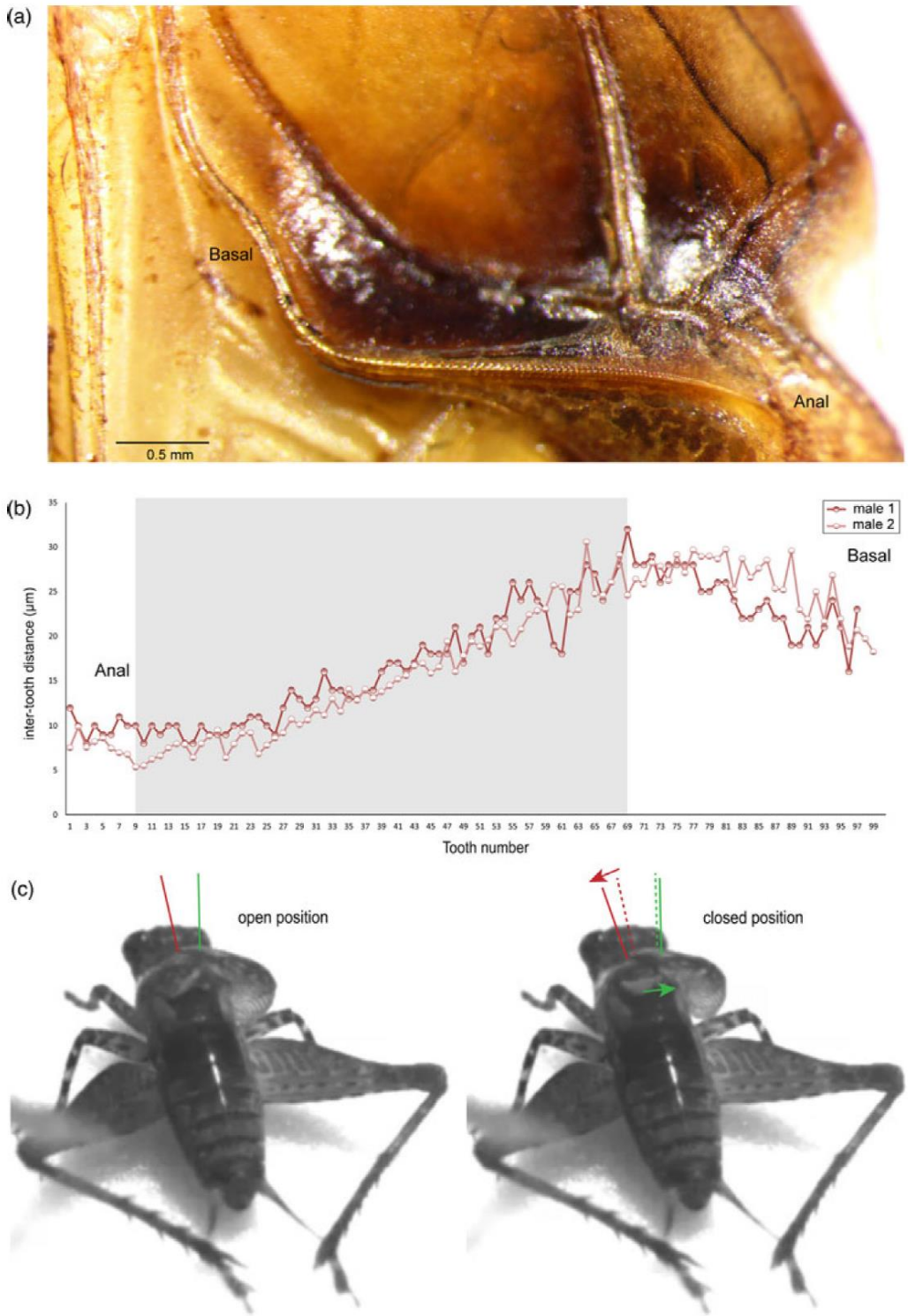

Figure 3. (Colour online) Stridulation of *L. fuscus*. Photograph of the stridulatory file in ventral view (a). File inter-tooth spacing measured in two specimens (shown in different colours); the grey area highlights the file region used for sound production (b). Snapshots from HSV recording of a singing male (c); FWs in open (left) and closed positions (right), with lines indicating the inner margin of each FW: a plain line represents the current position of the FWs, whereas dotted lines indicate the previous location (red: right FW, green: left FW); arrows indicate the closing direction of each wing (the stridulatory movement for one echeme is shown in Supplementary Video 2).

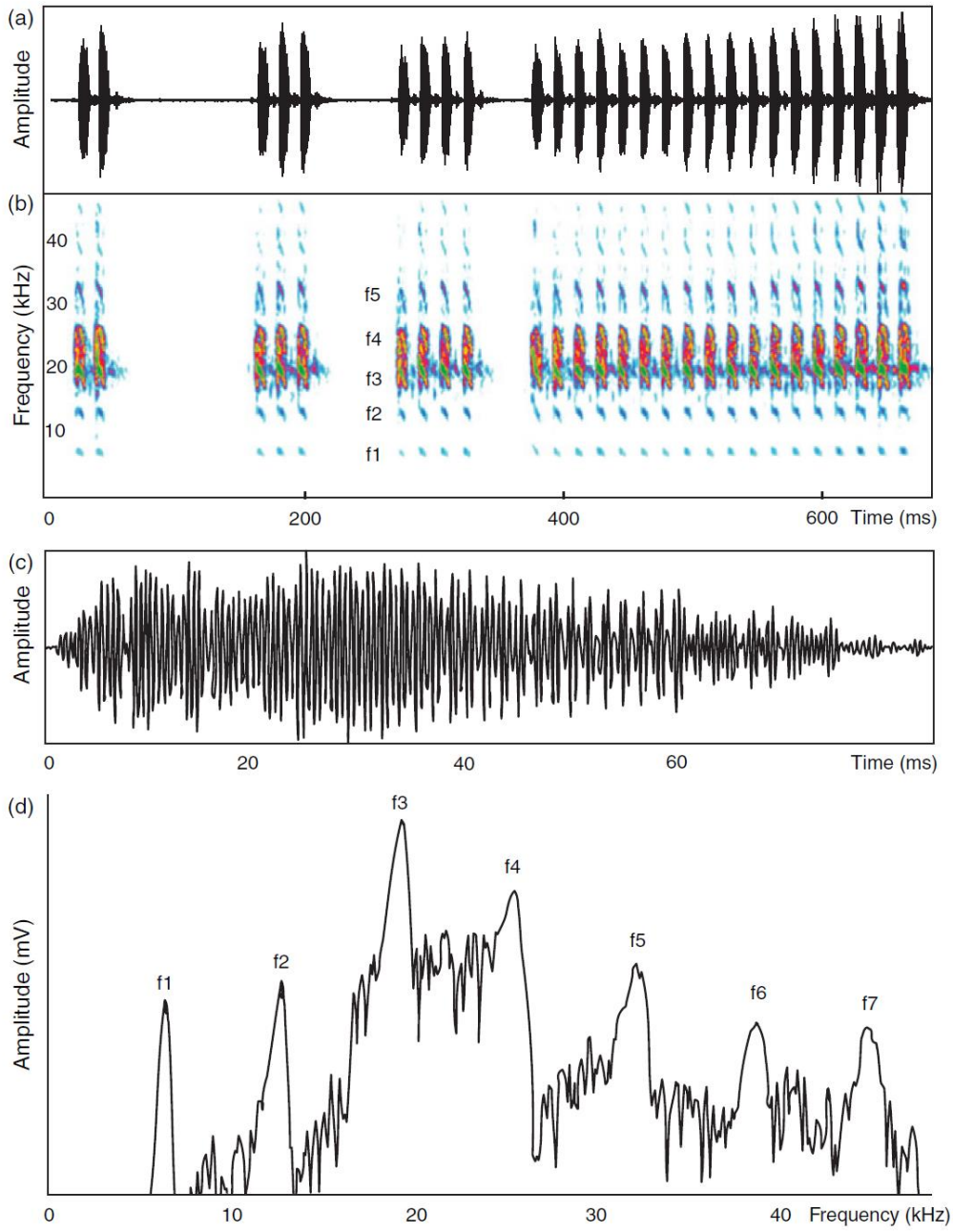

Figure 4. (Colour online) Calling song of *L. fuscus*. Oscillogram (a) and sonogram (b) of a complete echeme; oscillogram of a syllable (c); linear frequency spectrum from a syllable (d).

corresponds with the speed of teeth–plectrum interaction, which can help with determining the fundamental frequency.

Under the null hypothesis that TRS produces the lowest peak in the spectrum, the instantaneous speed of the system would be 103 mm/s, whereas it would be 314 mm/s if TSR produced the third (dominant) peak. The global estimated speed from syllable duration is 121 mm/s, which matches the null hypothesis that TSR is the LF peak of the spectrum, i.e. that the tooth impact generates the LF and not the dominant peak at HF. As a

consequence, one tooth impact would generate three primary waves due to the vibratory properties of the resonator, and the fd is a harmonic of the LF fundamental peak.

P. venosa

The stridulatory file of this species is very long and composed of ca. 370 teeth in the studied male. As in *L. fuscus*, the file structure and the teeth are simple. We estimated the functional part to consist of ca. 266 teeth, with a mean inter-tooth distance of 7.14 ± 0.55 μm (tooth density = 140/mm) (Figure 5(a)).

The song of *P. venosa* consists of a single long syllable that includes silent gaps (Figure 6(a),(b)), probably caused by jerking movements during FW closure. These monosyllabic calling songs last 93.25 ± 3.85 ms with a syllable period of 7.1 ± 2.4 s. The power spectrum contains spectral peaks at 6.0 ± 0.1, 11.6 and 18.0 kHz, the latter co-dominating with the fundamental frequency (but see discussion) (Table 4; Figure 6(d)). The sonogram (Figure 6(c)) demonstrates that the song spectrum actually differs between the first and second half of the song. First half of the syllable has three frequency peaks with clear harmonic structure (approx. 6, 12 and 18 kHz), whereas the second half of the syllable has slightly HFs and also an additional peak between 12 and 18 kHz, with no apparent harmonic basis.

Given the under-sampling of HFs revealed in this study for the previous recordings of *L. fuscus* compared with recordings made with appropriate equipment, it is likely that the third frequency peak of *P. venosa* is also under-sampled in the recording analysed here. Under this assumption, the song of *P. venosa* would be dominated by HFs as documented in *L. fuscus*.

The hypothesis that one FW closure produces one long syllable seems the most likely to explain the song because the silent gaps observed within the song are too short and heterogeneous to represent FW openings. Under this hypothesis, the monosyllabic song would be produced by a slow FW closure with a global speed estimated at 20.5 mm/s.

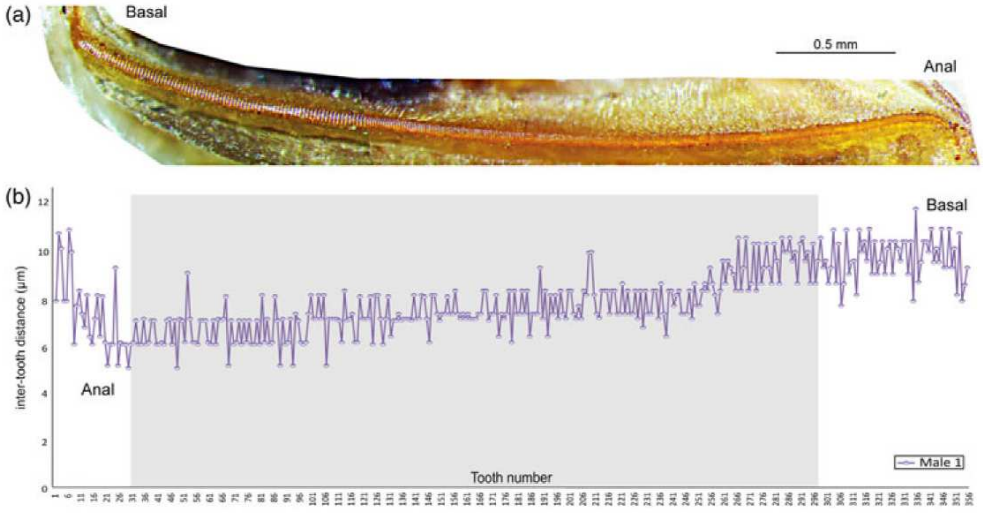

Figure 5. (Colour online) Stridulatory file of *Ponca venosa*. Photograph of the stridulatory file in ventral view (a). File inter-tooth spacing measured in one male; the grey area highlights the file region used for sound production (b).

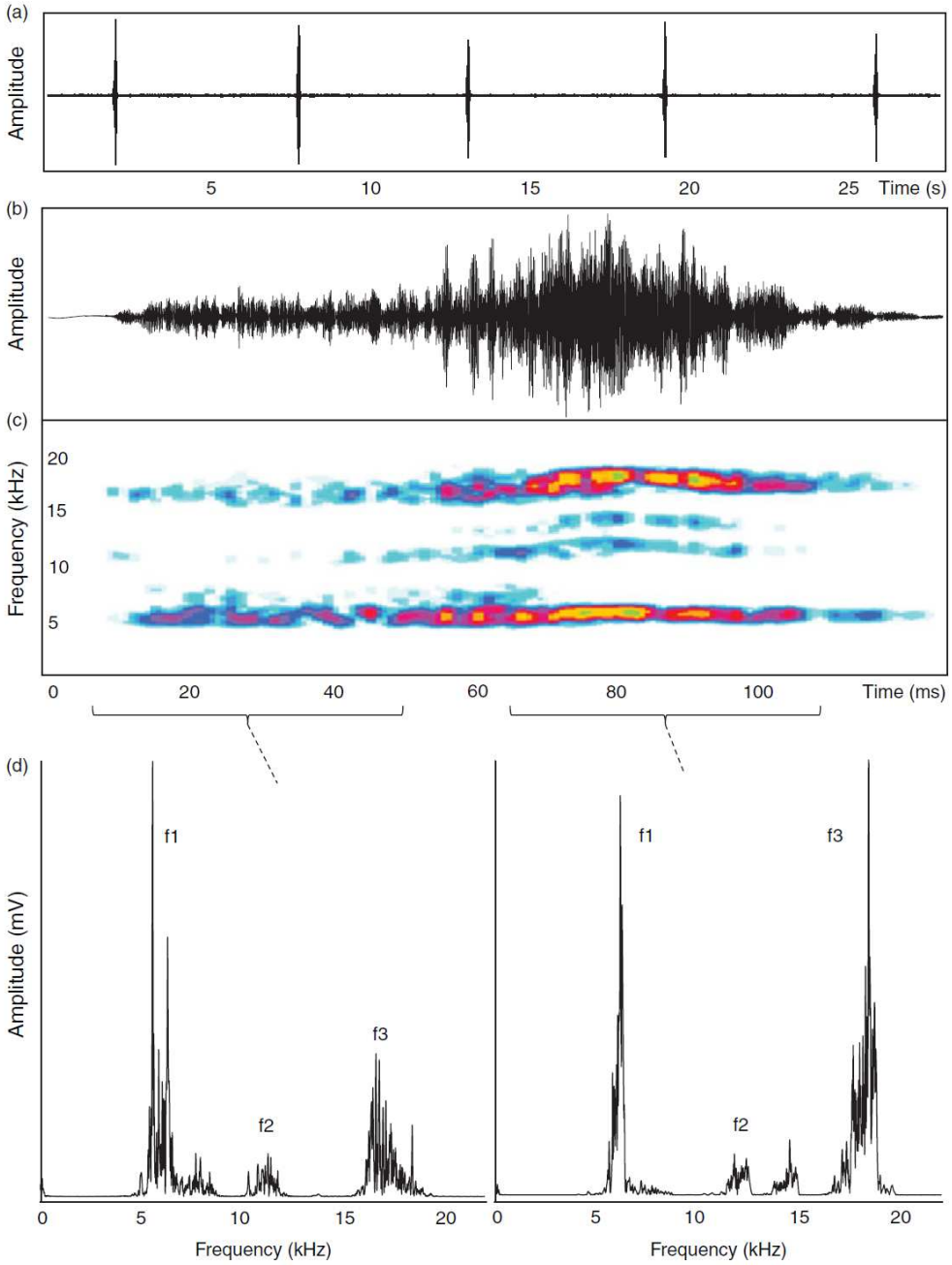

Figure 6. (Colour online) Calling song of *Ponca venosa*. Oscillogram of a bout of five syllables (a); oscillogram (b) and sonogram (c) of one syllable, linear frequency spectrum of the first 50 ms of the syllable (d) and the last 50 ms of the syllable (e).

This is an underestimate because FW closure is not continuous, but includes stops resulting in gaps within the song.

Under the null hypothesis that TSR produces the lowest frequency of the spectrum, the instantaneous speed of the system is estimated to be 42 mm/s, whereas it is estimated to be 128 mm/s if TSR corresponds with the third (co-dominating) peak. Although

underestimated, the global speed from syllable duration (20.5 mm/s) supports the null hypothesis that the tooth impact generates the LF and not the co-dominant (likely dominant) peak at HF. As a consequence, as in *L. fuscus*, one tooth impact would generate three primary waves due to the vibratory properties of the resonator. In addition, the ratio between instantaneous and global estimated speeds allows us to predict that the gaps within the syllables increase the syllable duration by a factor of 2.05. In other words, without the pauses made by the FWs during the closing movement, the song of *P. venosa* would be likely to last ca. 45.50 ms instead of 93.25 ms.

Discussion

Cricket songs are typically described as LF, pure-tone signals (e.g. Michelsen 1998), although most contain low-amplitude, HF harmonics. Because these harmonics are much less intense than the fundamental frequency of the song, they are often considered unimportant to the behaviour of crickets (Dumortier 1963; Leroy 1966; Balakrishnan and Pollack 1996; Robinson and Hall 2002; Hung and Prestwich 2004), and there has been less work done on the biological significance of these HF harmonics than the fundamental frequency. However, several authors have suggested that these HF components might be detected and used by females for phonotaxis at close range (Latimer and Lewis 1986) or play a significant role in courtship behaviour, when these HF components are often relatively greater than during calling song (Hoy 1991; Libersat et al. 1994). As we show here, the type of equipment used during recordings can have a significant impact on characterizing the relative amplitude of these harmonics and many cricket species might have considerable, undetected variation in the relative amplitudes of their HF harmonics (T. Robillard, pers. obs.; see Supplementary Figure 1).

In the subfamily Eneopterinae, recent studies have demonstrated that HFs are likely to represent a key evolutionary novelty enabling distinctive acoustic communication (Robillard and Desutter-Grandcolas 2004a). Clearly, as data accumulate, it becomes clear that these crickets rely on HFs to communicate. About 250 species are currently known in this subfamily (Eades et al. 2014; T. Robillard, pers. obs.), but only ca. 50 species have been recorded with equipment capable of detecting HF components. Nevertheless, among those, most species proved to use calling songs with HFs. In particular, the whole tribe Lebinthini, speciose and diversified in the Southwest Pacific and Southeast Asia, uses HF harmonics as fds, whether as derived fundamental peaks or as real harmonics (Robillard and Desutter-Grandcolas 2004a; Robillard et al. 2007, 2013). But even in the species with LF songs, such as *Nisitrus vitattus* (Haan, 1842), the harmonics carry significantly high energy relatively to the dominant peak (Robillard et al. 2013). This led to the hypothesis that having harmonics with enhanced radiating power is ancestral in these crickets, and that it appeared before the harmonics were used as the fd. The advantages of using songs with HFs, either as fd or as subdominant harmonics, are still to be determined, although several hypotheses have been proposed before in relation with the action of integrated factors of selection (predation, sexual selection, interspecific competition for acoustic space, population structure, etc.). Hypothesis explaining the rise of HFs in these species also consider the differential attenuation and propagation of the different components of the spectrum, which may have consequences on phonotaxis (Römer 1993; Robillard and Desutter-Grandcolas 2004a; Robillard et al. 2007).

In this study, we analysed the songs of the three species representing the three eneopterine genera present in the Neotropics in order to evaluate whether they also possess important HF components. The results show that all eneopterine genera from the

Neotropics also have species that use HFs to communicate, based on the rich harmonic content of their songs. Strikingly, the results suggest that these genera do not present a spectral pattern like in *N. vittatus*, in which the LF fundamental is the fd. The Neotropical eneopterines possess high fds, recalling the patterns observed in the tribe Lebinthini, with dominant harmonic peaks for *Ligypterus* and *Ponca*, while *Eneoptera* species possess unique features (see below). The three studied species highlighted, however, differences in their use of these HFs. Below, we discuss the new findings for each of them.

For *E. guyanensis*, the HSV recordings confirm the complex stridulatory behaviour hypothesized by Robillard and Desutter-Grandcolas (2011a) (Supplementary Video 1): each sound pulse of the song is generated by a very short opening–closing movement, involving alternately the normal region of the file to produce the "LF" syllables of the song, and the region with double teeth, producing the "HF" syllables. The two parts are used as if they were separate stridulatory files, except during the transitions between the LF and HF parts of the song.

The new findings for this species are mostly due to the new recordings made with equipment allowing for a proper sampling of HFs. Their spectral analysis reveals that the song is entirely driven by HFs (Figure 1). Even during the part of the song that was previously considered as "LF", the fifth harmonic is either co-dominating or dominating over the lower fundamental frequency generated by the simple teeth (Figure 1(c)). This fifth harmonic is nearly the same frequency (ca. 20 kHz) as the maximum of the broadband sound generated when the double teeth are used to make the louder, HF part of the song, which lacks an LF component. In other words, these data suggest that the harp of this species resonates mostly at HF, but each region of the file induces resonance by two different mechanisms, either through the harmonics of low fundamental frequency (slow TSR), or as a new fundamental frequency (quick "crest"-impact rate), produced by the crests of the double teeth. This is consistent with the hypothesis that eneopterines tend to exploit pre-existing vibrational capacities of the harp corresponding to the harmonics from lower fundamental frequencies (Robillard et al. 2013).

This finding also supports the hypothesis of convergent evolution for HF calling in Eneopterinae. Although it is clear that the case of *Eneoptera* demonstrates a new example of true HF acoustic communication in crickets, it is also clear that there are differences in bioacoustic features of the HFs found in *E. guyanensis* and the Lebinthini, where the use of HFs is widespread. Such differences might result from phylogenetically independent pathways, as hypothesized before (Robillard and Desutter-Grancolas 2004a) and confirmed by molecular phylogenetic studies (Robillard and Desutter-Grandcolas 2006; Nattier et al. 2011). This may be due to pre-existing capacities of the whole subfamily to use communication channels corresponding to HFs. Given these results, it is reasonable to speculate that HF songs are more common in eneopterine species than previously thought. Particular attention should be directed at other species in the genus *Eneoptera* that do not have double-teeth structures, such as *E. surinamensis*. The song of this species was briefly described in the taxonomic revision of the Neotropical eneopterines (Robillard and Desutter-Grandcolas 2005) and later by Miyoshi et al. (2007), who demonstrated that the song comprised only LF syllables, but of two types with different durations, and that the file included only normal teeth, as in the LF part of the song of *E. guyanensis*. However, the recordings used in these previous studies did not allow any estimation of the HF content of their spectrum. If the LF syllables of *E. surinamensis* are comparable with that of *E. guyanensis*, we predict that a large part of the spectrum might have been overlooked, and that this species is a good candidate as another case of a HF singing cricket.

In *P. venosa* and *L. fuscus*, our study suggests that the fd of the song is the third harmonic of the spectrum. Clearly, the spectrum presented before for *L. fuscus* and another species of the genus, *L. linharensis* (Robillard, 2005), were wrongly established, based on under-sampled recordings that attenuated the HF part of the song, as would a low-pass frequency filter. The current recordings for *Ponca* have the same issues, with obviously under-sampled HFs. Therefore, it is likely that the third frequency peak of *P. venosa,* which appears here as co-dominating, is in fact highly under-sampled in the recording analysed here. Under this assumption, the song of *P. venosa* would be dominated by HFs as documented in *L. fuscus*. As a consequence, as in *L. fuscus*, one tooth impact would generate three primary waves due to the vibratory properties of the resonator. The results suggest that the TSR would generate the LF of the spectrum, while the harp would vibrate at a HF corresponding to the third harmonic of the fundamental peak. This pattern is similar to that generalized in the genus *Cardiodactylus* (Robillard et al. 2007), which is found in Southeast Asia and in many archipelagos of the West Pacific (see Robillard 2009; Robillard and Ichikawa 2009; Tan and Robillard 2014; Robillard et al. 2014 for examples of spectra for this speciose genus).

As for song temporal patterns, the present data also suggest more similarities between the clade *Ponca–Ligypterus* with the Lebinthini than with *Eneoptera*. Rhythms are very different between the three Neotropical genera: both documented species of *Eneoptera* (*E. guyanensis* and *E. surinamensis*) show continuous trills, which have not been documented in the Lebinthini. However, the two other genera possess temporal patterns found in the Lebinthini: the monosyllabic song found in *P. venosa* is a very common pattern in *Cardiodactylus*, while the mixed temporal pattern of *Ligypterus* recalls the song of several *Lebinthus* or *Agnotecous* species, which often produce short trills initiated by spaced syllables (e.g. *Lebinthus bitaeniatus* Stål, 1877, *Lebinthus Luae* Robillard and Tan, 2013 – see Robillard and Tan 2013).

This series of evidence is consistent with the recent phylogenetic results, which suggest that *Ponca* and *Ligypterus* would form a clade closely related to the Pacific Lebinthini tribe, including *Cardiodactylus* and the other clades of eneopterines with HF songs (Nattier et al. 2011). The acoustic data presented here support the hypothesis that Neotropical species of Eneopterinae may have multiple origins, or at least that the clade *Ponca–Ligypterus* is more acoustically related to the Lebinthini tribe than to the sympatric genus *Eneoptera*. The multiple origins of Eneopterinae in South America suggested by these results will have to be confirmed in the future with more molecular data and taking into account biogeographical explanations for their distribution.

Conclusion

Crickets have been documented in bioacoustical studies for decades, but little attention has been given to the HF part of their songs, which are usually faint and often considered of little importance for communication. Our study shows, however, that HF components of cricket calling songs may have been overlooked by previous workers, especially in groups prone to HF singing, where such features may represent a significant part of acoustic diversity. HF components are clearly dominant in the species analysed here, but they could barely be detected with recordings made with equipment typically used to record cricket songs. In one case (*E. guyanensis*), this blurred the analysis by suggesting that a part of the song was LF only. In the case of *L. fuscus* (and probably in *P. venosa*), this limitation lead to the misinterpretation that these species were "normal" crickets in terms of bioacoustics, whereas they actually produce HF or ultrasonic calls to communicate. We thus

recommend cautious use of recordings made under the assumption that all crickets have a stereotyped LF calling song, as this assumption has not been tested in most clades of crickets.

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Supplementary material

Supplementary material for this article is available via the supplementary tab on the article's online page at http://dx.doi.org/10.1080/09524622.2014.996915

Notes

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