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Phylogeny of world stag beetles (Coleoptera: Lucanidae) reveals a Gondwanan origin of Darwin's stag beetle

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ABSTRACT

Stag beetles (family Lucanidae Latreille, 1804) are one of the earliest branching lineages of scarab beetles that are characterized by the striking development of the male mandibles. Despite stag beetles' popularity among traditional taxonomists and amateur collectors, there has been almost no study of lucanid relationships and evolution. Entomologists, including Jeannel (1942), have long recognized resemblance between the austral stag beetles of the tribes Chiasognathini, Colophonini, Lamprimini, Pholidotini, Rhyssonotini, and Streptocerini, but this hypothesis of their close relationship across the continents has never been tested. To gain further insight into lucanid phylogeny and biogeography, we reconstructed the first molecular phylogeny of world stag beetles using DNA sequences from mitochondrial 16S rDNA, nuclear 18S and 28S rDNA, and the nuclear protein-coding (NPC) gene *wingless* for 93 lucanid species representing all extant subfamilies and 24 out of the 27 tribes, together with 14 representative samples of other early branching scarabaeoid families and two staphyliniform beetle families as outgroups. Both Bayesian inference (BI) and maximum likelihood inference (MLI) strongly supported the monophyly of Lucanidae *sensu lato* that includes Diphyllostomatidae. Within Lucanidae *sensu stricto*, the subfamilies Lucaninae and Lampriminae appeared monophyletic under both methods of phylogenetic inferences; however, Aesalinae and Syndesinae were found to be polyphyletic. A time-calibrated phylogeny based on five fossil data estimated the origin of crown group Lucanidae as circa 160 million years ago (MYA). Divergence between the Neotropical and Australasian groups of the Chiasognathini was estimated to be circa 47 MYA, with the South African Colophonini branching off from the ancient Chiasognathini lineage around 87 MYA. Another Gondwanan relationship was recovered between the Australasian *Eucarteria* and the Neotropical *Casignetus*, which diverged circa 58 MYA. Lastly, as Jeannel's hypothesis predicted, divergence within Lampriminae between the Australasian *Lamprima* and the Neotropical *Streptocerus* was estimated to be circa 37 MYA. The split of these lineages were generally concordant with the pattern of continental break-up of the super-continent Gondwana, and our biogeographic reconstructions based on the dispersal-extinction-cladogenesis model (DEC) corroborate our view that the divergences in these austral lineages were caused by vicariance events following the Gondwanan break-up. In addition, the phylogenetic position and geographic origin of the Hawaiian genus *Apterocyclus* was revealed for the first time. Overall, our results provide the framework toward studying lucanid relationships and divergence time estimates, which allowed for more accurate biogeographic explanations and discussions on ancestral lucanids and the evolutionary origin of the enlarged male mandibles.

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1. Introduction

Stag beetles (family Lucanidae Latreille, 1804) belong to a unique lineage of scarab beetles characterized by the prolific evolution of the male mandibles. As a relatively small group within the superfamily Scarabaeoidea Latreille, the family Lucanidae contains about 1300 species distributed throughout all main zoogeographical regions except Antarctica (Benesh, 1960; Didier and Séguy, 1953; Fujita, 2010; Krajick, 2001; Mizunuma and Nagai, 1994). Due to their enormous size and ornamental mandibles, stag beetles have long received great attention from traditional taxonomists and amateur collectors. Nevertheless, despite extensive research on their taxonomy and classification, the phylogeny and evolutionary history of these beetles remain largely unknown. Holloway (1969, 1960) studied the lucanid phylogeny based on various

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morphological characters such as the male genital structure and ocular canthus. Her studies established the basis for the lucanid classification at the subfamilial level, but a phylogenetic component was mostly absent in her works. More recently, Hosoya et al. (2003) and Hosoya and Araya (2005) used the mitochondrial cytochrome oxidase subunit I (COI) and 16S ribosomal DNA (16S) sequences, respectively, to infer the phylogenetic relationships among stag beetle species in Japan. Their studies were the first to use molecular data to reconstruct a lucanid phylogeny, but both studies considered small data sets with very sparse coverage of the family (only 9 and 10 species, respectively, nearly all from Japan).

While entomologists have long recognized a resemblance between austral beetle taxa, including some lucanids, for more than a century (e.g., Erichson, 1842; Mackerras, 1925; Tillyard, 1926; reviewed by Cranston, 2009), there were only a few instances where vicariance associated with the Gondwanan break-up could explain beetle groups with Southern Hemisphere disjunction distribution (Table 1). Two alternative explanations include dispersal across oceans or contraction of formerly broader ranges. Many beetle groups that show an austral disjunction pattern were found to be too young to be influenced by the continental break-up of Gondwana and hence the trans-oceanic dispersal explanation has been accepted to explain the distribution of these lineages. On the other hand, a contraction explanation refers to once vagile or cosmopolitan lineages whose current distributions have been reduced to the Southern Hemisphere (e.g., Scolytinae; Sequeira and Farrell, 2001). In the family Lucanidae, both vicariance and dispersal explanations have been proposed to explain the biogeography of austral stag beetles. The first hypothesis was proposed by Holdhaus (1929) who argued that the disjunct distribution of closely related lucanids in the Southern Hemisphere was caused by a contraction in a formerly widespread distribution in both hemispheres during the Tertiary. Holdhaus (1929) used the presence of a fossil species *Palaeognathus succini* Waga from the northern Oligocene Europe as evidence for his hypothesis. While Holdhaus' (1929) argument was widely accepted (e.g., Ander, 1942; Brink, 1956; Chalumeau and Brochier, 2007, 2001; Landin, 1955), Jeannel (1942) challenged this theory in his discussion of the Paleantarctic origin of faunas in the Southern Hemisphere, in which he hypothesized a Gondwanan origin for the subfamily Chiasognathinae *sensu* van Roon (1910) (herein, the 'Jeannel's hypothesis').

As lucanid classification remains a subject of debate, the definition of Chiasognathinae has often been misused or misinterpreted. In fact, the Chiasognathinae is no longer a valid taxonomic group, but it is now comprised of four Neotropical genera, six Australasian genera, and *Colophon* of South Africa (Holloway, 1960; Jeannel, 1942). Even though Jeannel (1942) explicitly named only *Chiasognathus* and *Colophon* in his original text, Holloway (1960) suggested that Jeannel's hypothesis includes the following genera: *Pholidotus* (=*Casignetus*), *Chiasognathus, Sphaenognathus, Dendroblax, Rhyssonotus*, *Homolamprima*, *Cacostomus*, *Lamprima* (including *Neolamprima*), *Phalacrognathus, Streptocerus*, and *Colophon*. Under the current classification scheme, these genera can be grouped into six tribes of two subfamilies: Chiasognathini (*Chiasognathus* + *Sphaenognathus*), Pholidotini (*Casignetus*), Rhyssonotini (*Rhyssonotus* + *Cacostomus*), and Colophonini (*Colophon*) of the subfamily Lucaninae; and Lamprimini (*Lamprima* + *Hololamprima* + *Dendroblax* + *Phalacrognathus*), and Streptocerini (*Streptocerus*) of the Lampriminae. Based on morphological resemblance among these lineages and their presence on the old Australian islands (e.g., Lord Howe and Norfolk Islands), Jeannel (1942) predicted that these austral lineages had originated in Gondwana with some lineages later migrating into the Northern Hemisphere, leaving a fossil in Europe. Moreover, he suggested that the South African *Colophon* must be the oldest lineage within the 'Chiasognathinae.'

Interest and controversies concerning the biogeography of the Chiasognathini and other austral stag beetles was recently revived with the discovery of two *Sphaenognathus* species from the tablelands of Northeastern Australia (Moore and Monteith, 2004; Moore, 1978). The tribe Chiasognathini includes a species famously known as Darwin's stag beetle, *Chiasognathus grantii* Stephens, for its reference in *The Descent of Man, and Selection in Relation to Sex* (Darwin, 1871), and the discovery of a predominantly Neotropical genus *Sphaenognathus* from Australia certainly strengthened the possibility of their Gondwanan origin. Conversely, however, the second fossil species of 'Chiasognathinae,' *Protognathinus spielbergi* Chalumeau and Brochier, was discovered in the Eocene Messel oil Shale of Hesse, Germany (Chalumeau and Brochier, 2001), which lends support to Holdhaus' (1929) idea of a formerly worldwide distribution of Chiasognathini, that has been reduced to the current distribution in the Southern Hemisphere. As is evident in their subsequent publication *The Chiasognathinae of the Andes* (Chalumeau and

Table 1
Summary of studies that have tested for Gondwanan vicariance in beetle groups with disjunction distribution in the Southern Hemisphere.

Taxa	Taxonomic rank	Explanation	Evidence	Reference
Adephaga				
Scaritinae (Carabidae)	Subfamily	Dispersal	Molecule & morphology	Hogan (2012)
Migadopini (Carabidae)	Tribe	Vicariance	Morphology	Roig-Juñent (2004)
Aciliini + Eretini (Dytiscidae)	Tribe	Vicariance	Molecule	Bukontaite et al. (2014)
Polyphaga: Staphyliniformia				
Solieriinae (Staphylinidae)	Subfamily	Contraction	Fossil	Thayer et al. (2012)
Hydraenopsis (Hydraenidae)	Genus	Dispersal	Molecule	Trizzino et al. (2013)
Hydrophilidae	Family	Contraction	Molecule & morphology	Fikáček et al. (2014), Short and Fikáček (2013)
Cetiocyon (Hydrophilidae)	Genus	Dispersal	Morphology	Fikáček and Short (2010)
Polyphaga: Scarabaeiformia				
Canthonini + Dichotomiini (Scarabaeidae)	Tribe	Dispersal	Molecule	Philips et al. (2004), Sole and Scholtz (2010)
Omorginae (Trogidae)	Subfamily	Vicariance	Molecule	Strümpher et al. (2014)
Polyphaga: Elateriformia				
Arrhipis (Eucnemidae)	Genus	Vicariance	Molecule & morphology	Brüstle et al. (2010)
Metriorrhynchini (Lycidae)	Tribe	Dispersal	Molecule	Sklenarova et al. (2013)
Polyphaga: Cucujiformia				
Gymnochilini (Trogossitidae)	Tribe	Dispersal	Morphology	Leschen and Lackner (2013)
Clytrini (Chrysomelidae)	Tribe	Vicariance	Morphology	Agrain and Roig-Juñent (2011)
Scolytinae (Curculionidae)	Subfamily	Contraction	Molecule	Sequeira and Farrell (2001)

Brochier, 2007), the authors misused the term 'Chiasognathinae' in referring to the tribe Chiasognathini. Moreover, the characters that Chalumeau and Brochier (2001) used to place the fossil species *P. spielbergi* into the 'Chiasognathinae' (i.e., "tête moins large que le pronotum, prothorax non contigu aux élytres, mandibules plus longues que la tête, tibias antérieurs larges, avec de fortes épines sur la marge externe") are equivocal and often present in various other groups of Lucanidae. In fact, several characters discernable in the illustration from the original description, such as straight or subgeniculate antennae, antennal club with three antennomeres, and largely tridentate protibiae, even suggest its close affinity with the Lampriminae (Paulsen, 2010).

To test the potential Gondwanan origin of the austral stag beetles (in response to Jeannel's hypothesis) and to establish the basis for a lucanid phylogeny and classification, we have undertaken a comprehensive molecular study of the world Lucanidae based on mutilocus DNA sequence data. Because the Scarabaeoidea is often considered a relatively young lineage among beetles (e.g., Krell, 2006; Théodoridés, 1952), its radiation driven by continental drift has often been challenged. Nevertheless, as one of the earliest branching lineages of the Scarabaeoidea, the Lucanidae remains most likely to have been influenced by the Gondwanan break-up. By combining Bayesian divergence time estimates with biogeographic reconstruction of ancestral ranges, here we evaluate Jeannel's biogeographic scenarios and discuss the potential origin of the Lucanidae, together with its mandible evolution.

2. Material and methods

2.1. Taxon sampling

We sampled 93 species of Lucanidae (ingroup), plus as outgroups three species of Geotrupidae Latreille (Geotrupinae + Bolboceratinae), two species of Passalidae Leach (Passalinae + Aulacocyclinae), two species of Glaresidae Kolbe, and one representative species each of Diphyllostomatidae Holloway, Hybosoridae Erichson, Ochodaeidae Mulsant and Rey, Scarabaeidae Latreille, Trogidae MacLeay, Silphidae Latreille, and Histeridae Gyllenhaal following the classifications of Lawrence and Newton (1995) and Smith (2006). For the Lucanidae, 86 newly sampled species and seven species obtained from GenBank represent all four extant subfamilies, 24 out of the 27 tribes, 47 genera, and a broad sample of subgenera. Because the higher taxonomy within the Lucanidae remains controversial, particularly at the tribal level (Smith, 2006), we combined the classifications of Holloway (1997, 1968), Howden and Lawrence (1974), and Maes (1992a, 1992b). The problems with nomenclature for the tribes described by Benesh (1960) and Maes (1992a, 1992b) have been recognized (Smith, 2006), but we tentatively adopted these tribe names as they represent relatively robust phylogenetic groups. Voucher specimens and their extracted genomic DNA are deposited in the research collection at the Harvard University Museum of Comparative Zoology in Cambridge, MA, U.S.A.

2.2. DNA extraction, amplification, and sequencing

Specimens for DNA were mostly collected as adults and preserved either dried or in 100% ethanol. Total genomic DNA was extracted from either the thoracic muscle, flight muscle, one or more legs, or from the entire specimen, by grinding or soaking, using the QIAquick DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. We maximized use of museum specimens by adopting a non-destructive extraction method, and by reducing the amount of elution buffer to 150 μl, which yielded high-concentration genomic DNA. PCR amplification was typically conducted in 25 μL reactions containing 11.25 μL HPLC H_2O, 2.5 μL 5X buffer, 1.5 μL $MgCl_2$, 5 μL Q solution (except for WNG), 0.5 μL 10 mM dNTPs, 0.25 μL Taq DNA polymerase (all from Qiagen, Germany), 1.5 μL each primer (10 mM), and 1 μL of genomic DNA. In addition, 0.25 μL ExTaq DNA polymerase (Takara, Japan) was used instead of the Qiagen Taq for 16S, and the amount of genomic DNA was adjusted up to 3 μL based on the condition of specimens, particularly for those from museums. For the final data set, we targeted approximately 2320 bp of double-stranded DNA sequence data for each specimen. Our data included approximately 989 bp of the mitochondrial ribosomal gene 16S, ~354 bp of 18S rDNA, ~505 bp of 28S rDNA, and ~447 bp of *wingless* (WNG).

16S was amplified in two shorter fragments using the paired primers 16SC and 16SD, and 16SA and 16SB (Hosoya et al., 2001), yielding a product of approximately 989 bp. Typical thermal cycling conditions for 16S included an initial denaturation step at 94 °C for 1:30 min, 40 cycles at 94 °C, 48 °C, 72 °C for 1 min each, and final extension at 72 °C for 3 min. 18S V7 region was amplified using the paired primers CV7F-1 and CV7R following the protocol in Raupach et al. (2010). The primer CV7F-1 was modified from CV7F (Raupach et al., 2010) based on the available 18S sequence data of the Lucanidae. 28S domains 3 to 5 (D3-D5) was amplified using the paired primers ZR1 (Mallat and Sullivan, 1998) and rd5b (Whiting, 2002), yielding a product of approximately 447 bp by following the protocol as described in Mckenna et al. (2014). WNG was amplified using the primers Wg550F and WgAbrZ (Wild and Maddison, 2008), followed by nested PCR using Wg578F (Ward and Downie, 2005) and WgAbR (Abouheif and Wray, 2002). Typical thermal cycling conditions for WNG included an initial denaturation at 94 °C for 2 min, 37 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 5 min. 1 μL of the PCR product from the first PCR was used for the second nested PCR. Primers used for amplification are summarized in Table 2.

Amplicons were purified using shrimp alkaline phosphatase and exonuclease I (USB Corp., USA) for 16S and WNG, or gel extracted and purified using the QIAquick Gel Purification Kit (Qiagen, Germany) for 18S and 28S. For sequencing, we used the ABI PRISM BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies, USA), and cycle sequencing reactions were performed on ABI PRISM 3130xl or 3730xl automated sequencers (Life Technologies, USA) at Harvard University. All sequences generated in this study were summited to GenBank under accession numbers KP250203-KP250535 as summarized in Table S1.

2.3. Nucleotide sequence alignment

Sequences were viewed, assembled and edited in Geneious version 7.0.5 (Biomatters Ltd., New Zealand). All sequences were aligned using the automatically selected algorithm in MAFFT version 7.017 with the default alignment parameters (scoring matrix: 200PAM/k = 2; and gap opening penalty: 1.53) (Katoh and Toh, 2008; Katoh et al., 2002). The resulting aligned sequence matrices for 18S and 28S were masked using Gblocks verison 0.91b (Castresana, 2000; Talavera and Castresana, 2007) with the less stringent parameters (min. number of sequences for a conserved position = 1/2 the number of sequences; min. number of sequences for a flank position = 1/2; max. number of contiguous nonconserved positions = 8; min. length of a block = 5; and allowed gap positions = "with half") to eliminate poorly aligned positions and divergent regions. The resulting alignment of a nuclear protein-coding gene WNG was first viewed and manually edited as nucleotides. Because this region of WNG does not contain introns (Wild and Maddison, 2008), we translated the entire alignment into amino acids in Geneious to edit incomplete codons either by inserting "N" or by trimming if found at the end of the sequence.

Table 2
Summary of oligonucleotide primers used in this study.

Locus	Primer name and direction	Primer sequence	Length	Reference
16S rDNA	16SC (sense)	5′-TACCTTGTGTATCAGGGTTTAT-3′	22 mer	Hosoya et al. (2001)
	16SA (sense)	5′-CGCCTGTTTAACAAAAACATGT-3′	22 mer	Hosoya et al. (2001)
	16SD (antisense)	5′-ATTATGCTACCTTTGCACGGTC-3′	22 mer	Hosoya et al. (2001)
	16SB (antisense)	5′-CCGGTTTGAACTCAGATCATGT-3′	22 mer	Hosoya et al. (2001)
28S rDNA D3–5	ZR1 (sense)	5′-GTCTTGAAACACGGACCAAGGAGTCT-3′	26 mer	Mallat and Sullivan (1998)
	rd5b (antisense)	5′-CCACAGCGCCAGTTCTGCTTAC-3′	22 mer	Whiting (2002)
18S rDNA V7	CV7F-1 (sense)	5′-CTTAAAGGAATTGACGGAAGGGCACCACC-3′	29 mer	Present study
	CV7R (antisense)	5′-GATTCCTTCAGTGTAGCGCGCGTG-3′	24 mer	Raupach et al. (2010)
Wingless	Wg550F (sense)	5′-ATGCGTCAGGARTGYAARTGYCAYGGYATGTC-3′	32 mer	Wild and Maddison (2008)
	Wg578F (sense)	5′-TGCACNGTGAARACYTGCTGGATG-3′	24 mer	Ward and Downie (2005)
	WgAbR (antisense)	5′-ACYTCGCAGCACCARTGGAA-3′	20 mer	Abouheif and Wray (2002)
	WgAbRZ (antisense)	5′-CACTTNACYTCRCARCACCARTG-3′	23 mer	Wild and Maddison (2008)

The WNG nucleotide alignment was once again translated into amino acids and re-aligned using the automatically selected algorithm in MAFFT with the default alignment parameters. Finally, this amino acid alignment was used to build a final nucleotide sequence alignment in TranslatorX (Abascal et al., 2010). All sequence alignments were concatenated in Phyutility version 2.2 (Smith and Dunn, 2008).

2.4. Data partitioning and model selection

A concatenated supermatrix of 2319 aligned nucleotide positions for 107 species was used in the program PartitionFinder version 1.1.1 (Lanfear et al., 2012) for selection of the best data partition scheme and best-fitting model selection. The total of three partitions were determined to be optimal using the greedy algorithm under the Akaike information criterion (AIC) with unlinked branchlengths: (1) 16S, (2) 18S, 28S and WNG 1st and 2nd codon positions, and (3) WNG 3rd codon position. We also searched partition schemes using the corrected Akaike information criterion (AICc) and the Bayesian information criterion (BIC). The AICc determined the same partitions as AIC, and the BIC produced only two partitions by grouping (2) and (3) as a single partition. Because it is more likely that the mitochondrial gene, nuclear ribosomal genes, 1st and 2nd codon positions, and 3rd position of a NPC gene to have different rates of evolution, we used a partition scheme determined by the AIC and AICc. Nevertheless, three trials of partitioned MLI analyses with these partition schemes produced nearly identical topologies (Figs. S1 and S2), suggesting that different partition schemes had trivial effects on our phylogenetic analyses. Finally, best-fitting models were selected for each partition under the AIC: The GTR + I + Γ model for (1) and (2), and TVM + Γ model for (3). Nevertheless, all phylogenetic analysis programs used in this study do not have the TVM model implemented and thus we used its closest model, the GTR + Γ model, for all analyses. Moreover, as there is some concern that the gamma distribution (Γ) already accounts for nearly invariable sites and hence conflicts with the invariable site parameter (I) (e.g., Gu et al., 1995; Mayrose et al., 2005; Sullivan et al., 1999; Yang, 1993), we instead adopted the GTR + Γ model for all partitions.

2.5. Phylogenetic analyses

Eight simultaneous runs, each with four metropolis-coupled chains, of unconstrained partitioned Bayesian analyses were executed in MrBayes v3.2.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with random starting trees on the Harvard University Faculty of Arts and Sciences Research Computing Odyssey cluster, using the aforementioned partitioning scheme and the GTR + Γ model with default priors. The analyses ran for 40,000,000 generations and trees were sampled every 10,000 generations. The resulting eight log files were evaluated in Tracer v1.6 (Drummond and Rambaut, 2007) and AWTY (Nylander et al., 2008), in which bivariate plots of the split frequencies for the two runs and the cumulative split frequencies were evaluated to diagnose both parametric and topological convergence. We combined trees after discarding the first 8 million generations as burn-in for each of the eight runs in LogCombiner v1.7.5, and generated the maximum clade credibility tree in TreeAnnotator v1.7.5. The resulting tree was then rooted with the two distant outgroups of Staphyliniformia. We interpreted BPP values $\geqslant$0.95 as strong support, BPP values $\geqslant$0.75 and $\leqslant$0.95 as moderate support, and BPP values $\leqslant$0.75 as weak support. Unconstrained Maximum Likelihood (MLI) rapid bootstrap analysis with 1000 replicates was performed in RAxML v7.2.8 (Stamatakis, 2006) using the GTR + Γ model with the same partitioning scheme as in BI. We interpreted Maximum Likelihood bootstrap (MLB) values $\geqslant$75% as strong nodal support, MLB values $\geqslant$50% and $\leqslant$75% as moderate support, and MLB values $\leqslant$50% as weak support.

2.6. Divergence time analysis

A time-calibrated tree was obtained under BI using Markov Chain Monte Carlo (MCMC) methods implemented in BEAST v2.1.3 (Bouckaert et al., 2014). To account for uncertainty in divergence time estimation, we employed an uncorrelated lognormal relaxed clock model (Drummond et al., 2006) and probabilistic fossil calibration priors. Monophyly constraints were placed on nodes with fossil calibrations, which were determined based on our phylogenetic analyses. The priors on the age of the nodes were set to a gamma distribution with offsets 5 MY younger than the minimum age of the fossil. Four independent MCMC runs with different seeds and random starting trees were executed on the Odyssey cluster for 40,000,000 generations, sampling every 10,000 generations. Convergence was once again diagnosed through graphical and statistical analyses on Tracer v1.6 (Drummond and Rambaut, 2007) and AWTY (Nylander et al., 2008). After discarding the first 8 million generations as burn-in, post burn-in samples from the four MCMC runs were combined in LogCombiner v1.7.5, which was used to generate the maximum clade credibility tree in TreeAnnotator v1.7.5.

Among approximately 24 species of fossil lucanids described to date, we conservatively selected five fossils for minimum-age calibrations (Table 3). Two fossil lucanids have been reported from the Jurassic, *Paralucanus mesozoicus* Nikolajev and *Juraesalus atavus* Nikolajev et al., where the former has later been erected to give rise to a separate family, Paralucanidae Nikolajev. Nevertheless, as Krell (2006) pointed out in his review of the fossil record and evolution of Scarabaeoidea, Nikolajev did not consider displacement of body parts or changes in size due to gaseous inflation during decay and fossilization, undermining the value of his classification. Hence, we

Table 3
Summary of fossil Lucanidae *s. l.* Fossils used for calibrations are marked with the corresponding node numbers as indicated in Fig. 2, and the dagger symbol (†) indicates extinct groups.

Family	Subfamily	Species	Author	Age and locality
†Paralucanidae	†Paralucaninae	*Paralucanus mesozoicus*	Nikolajev, 2000	L Jurassic, Shara-Teg, Gov'-Altai' Aymag, Mongolia
Lucanidae	†Protolucaninae	*Protolucanus jurassicus*	Nikolajev, 2007	L Jurassic, Anda-Zhuduk, Mongolia
Lucanidae	†Ceruchitinae	*Ceruchites hahnei*	Statz, 1952	Oligocene, Rott, Germany
Lucanidae	Aesalinae	*Juraesalus atavus***(1)**	Nikolajev et al., 2011	L Jurassic, Daohugou Village, Inner Mongolia; 159.8 ± 0.8 MYA (He et al., 2004)
Lucanidae	Aesalinae	*Sinaesalus tenuipes*	Nikolajev et al., 2011	E Cretaceous, Yixian Formation, Inner Mongolia; 129.7–122.1 MYA (Bai et al., 2012)
Lucanidae	Aesalinae	*Sinaesalus longipes*	Nikolajev et al., 2011	E Cretaceous, Yixian Formation, Inner Mongolia; 129.7–122.1 MYA (Bai et al., 2012)
Lucanidae	Aesalinae	*Sinaesalus curvipes*	Nikolajev et al., 2011	E Cretaceous, Yixian Formation, Inner Mongolia; 129.7–122.1 MYA (Bai et al., 2012)
Lucanidae	Aesalinae	*Cretaesalus ponomarenkoi*	Nikolajev, 1993	L Cretaceous, Kzyl-Zhar, Kazakhstan
Lucanidae	Syndesinae	*Prosinodendron krelli* **(2)**	Bai et al., 2012	E Cretaceous, Yixian Formation, Inner Mongolia; 129.7–122.1 MYA (Bai et al., 2012)
Lucanidae	Syndesinae	*Syndesus ambericus*	Woodruff, 2009	Late Eocene, Dominican Republic
Lucanidae	Syndesinae	*Ceruchus fuchsii* **(5)**	Wickham, 1911	Eocene, Florissant, USA
Lucanidae	Lampriminae	*Protognathinus spielbergi*	Chalumeau and Brochier, 2001	Eocene, Grube Messel, Germany
Lucanidae	Lucaninae	*Cretolucanus longus*	Nikolajev, 2007	E Cretaceous, Pad Semen, Russia
Lucanidae	Lucaninae	*Cretolucanus ordinarius*	Nikolajev, 2007	E Cretaceous, Pad Semen, Russia
Lucanidae	Lucaninae	*Cretolucanus sibericus*	Nikolajev, 2007	E Cretaceous, Pad Semen, Russia
Lucanidae	Lucaninae	*Succiniplatycerus berendti*	Zang, 1905	Eocene, Baltic amber
Lucanidae	Lucaninae	*Platycerus sepultus*	Germar, 1837	Oligocene, "in carbone fossili territorii Rheni prope Bonnam", Germany
Lucanidae	Lucaninae	*Platycerus zherichini*	Nikolayev, 1990	Oligocene, Pozhar, region, Russia
Lucanidae	Lucaninae	*Dorcus primigenius* **(4)**	Deichmüller, 1881	Eocene, Kutschlin near Bilin, Czech Republic
Lucanidae	Lucaninae	*Lucanus fossilis* **(3)**	Wickham, 1913	Eocene, Florissant, USA
Lucanidae	Lucaninae	*Miocenidorcus andancensis*	Riou, 1999	Miocene, Andance, France
Lucanidae	Subfamilia incerta	*Dorcasoides bilobus*	Motschulsky, 1856	Eocene, Baltic Amber
Lucanidae	Subfamilia incerta	*Paleognathus succini*	Waga, 1883	Eocene, Baltic Amber

have only selected fossil species that provide precise age and morphological characters that enable for accurate placement within the phylogeny.

The fossil of *Juraesalus atavus* was described from the Daohugou Bed of Inner Mongolia, China, which occurs on the boundary between the latest Middle Jurassic (Callovian) and the earliest Late Jurassic (Oxfordian), and has been dated to 159.8 ± 0.8 Ma (He et al., 2004). Nikolajev et al. (2011) considered this fossil to be a stem group aesaline, for it exhibits "antennae with 3-segmented non-lamellate club, mandibles produced beyond apex of clypeus, eyes not divided by canthus, and abdomen with 5 visible sternites." However, these characters are equivocal and commonly found throughout many groups of extant lucanids, such as *Ceruchus* and *Sinodendron* of the subfamily Syndesinae, as well as *Platyceropsis* and *Platyceroides* of the subfamily Lucaninae. Moreover, the extant aesalines often have short ocular canthi (e.g., *Aesalus*, *Lucanobium*, and *Trogellus* species). Hence, we considered this fossil as the oldest crown group lucanid (i.e., Lucanidae *sensu stricto*; node 1). In addition, the fossil of *Prosinodendron krelli* Bai et al. was described from the Yixian Formation of Inner Mongolia, China (Bai et al., 2012), which has been dated to 123.9 ± 1.1 MYA of the Lower Cretaceous (Yang et al., 2007). Based on the description and illustrations of the fossil, we were able to associate *Prosinodendron* with modern lucanids excluding *Aesalus*, and used it to calibrate the node shared by the Syndesinae, Lampriminae, Lucaninae, and an aesaline genus *Nicagus* (node 2).

Based on the affinities with the extant genera as determined in the original descriptions, three additional fossils were used to calibrate more recent nodes as illustrated in Fig. 2. First, *Lucanus fossilis* Wickham was described from the Florissant formation of Colorado, U.S.A., based on a fossil represented by an elytron, a scutellum and some fragments of prothoracic disc. Wickham (1913) considered this fossil to be closely related to extant *Lucanus dama* Fabricius (=*Lucanus capreolus* (Linnaeus)) and *Lucanus placidus* Say. Due to some uncertainty Wickham (1913) expressed about his taxonomic assignment, as well as insufficient morphological synapomorphies preserved, we assigned this fossil to *Lucanus s. l.*, which includes *Lucanus* and *Neosolucanus* (node 3). Second, *Dorcus primigenius* Deichmüller was described from Kutschiln near Bilin, Czech Republic, based on a fossil represented by a male head with distinct mandibles. Deichmüller (1881) considered this fossil to show affinities with extant *Eurytrachelus saiga* (Olivier) (=*Dorcus saiga*) and *Eurytrachelus platymelus* (Saunders) (=*Dorcus titanus platymelus*). Based on the illustration from the original description, we could associate *D. primigenius* with various extant subgenera of *Dorcus* (e.g., *Eurytrachelus*, *Serrognathus*, and *Macrodorcas*), and therefore, we assigned this fossil to represent the crown group *Dorcus* (node 4). Last, *Ceruchus fuschii* Wickham was described also from the Florissant formation of Colorado, U.S.A., and its original description suggests that it is closely related to extant *Ceruchus striatus* LeConte (Wickham, 1911). Since the morphology of *Ceruchus* is fairly conserved worldwide, we assigned *C. fuschii* to represent the crown group *Ceruchus* (node 5).

2.7. Biogeographic analyses

The biogeographic history of the Lucanidae was estimated under the dispersal-extinction-cladogenesis model (DEC) as implemented in Lagrange (Ree and Smith, 2008). As our interest in biogeography was restricted primarily to the austral stag beetle lineages associated with the fragmentation of Gondwana, we specified only five areas by considering the Palearctic, Nearctic, and Indomalaya regions as a single category of the Northern Hemisphere. Hence, our five-area biogeographic model divided the distribution of lucanids into the Northern Hemisphere/Holarctic, Australasian, Neotropical, Afrotropical, and Oceania regions. The outgroups were excluded from the analysis and the likeliest dispersal scenarios at all internal nodes were determined where the likeliness of a given scenario was represented by its

relative probability over a set of alternative scenarios. No constraint on adjacency matrix and the number of inhabitable geographic ranges by a lineage was specified, but the dispersal constraints were assigned in three time slices (0–35 MYA, 35–80 MYA, and 80–200 MYA) based on the connectivity of landmasses over time. Dispersal rates were assigned as 1.0 for contiguous areas, 0.1 for well-separated landmasses, and 0 for the Oceania region between 35 and 200 MYA since the Hawaiian Islands (the only Oceania region included in our analysis) did not exist during this time period.

3. Results

3.1. Phylogeny

The phylogeny under Bayesian inference (BI) (Fig. 1) generally showed more resolution with strong nodal supports than that under the maximum likelihood inference (MLI) (Fig. S1). These two phylogenies overall recovered the identical topology when the nodes with the Bayesian posterior probability (BPP) below 0.50 or the maximum likelihood bootstrap value (MLB) below

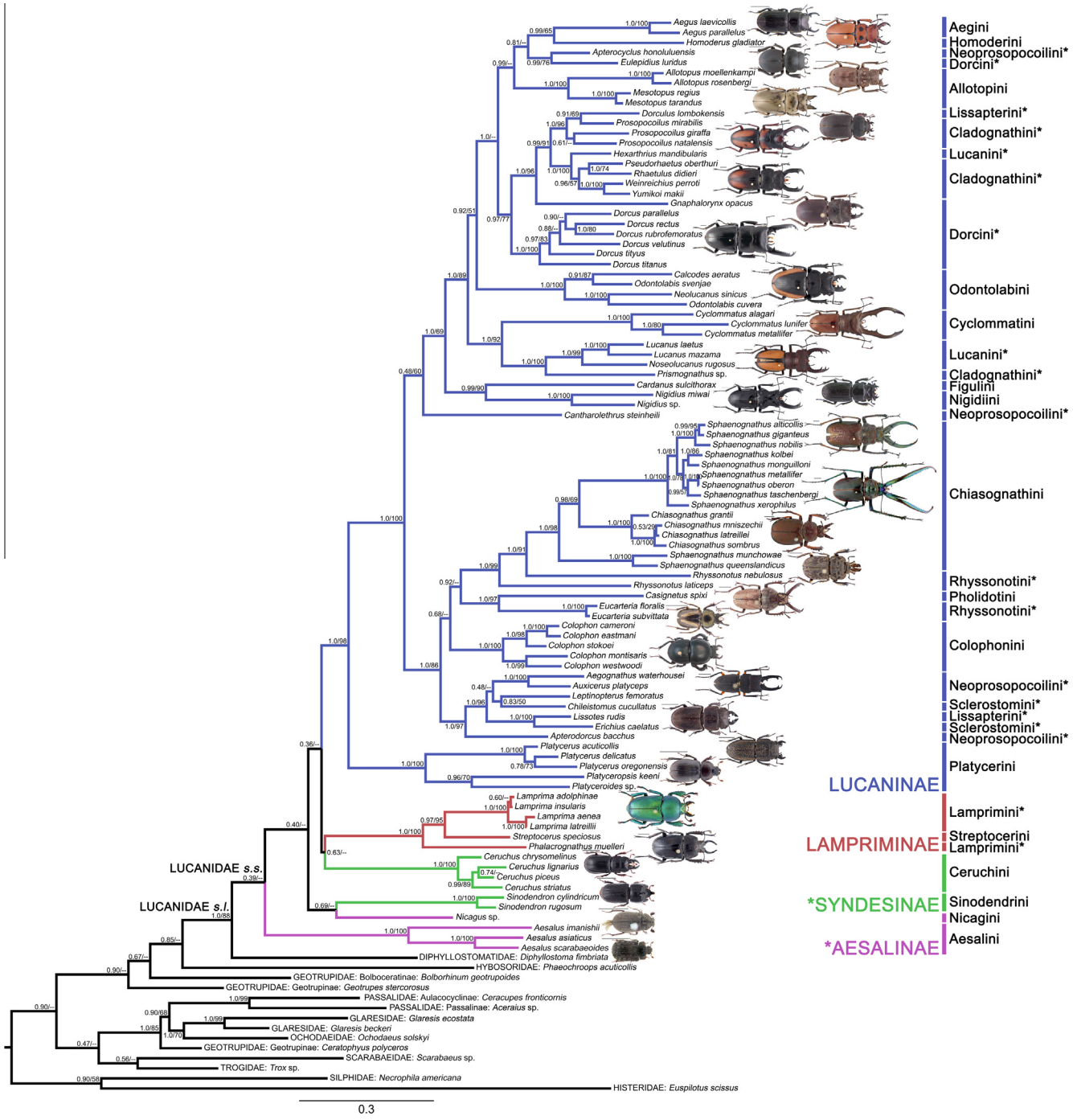

Fig. 1. Bayesian topology showing the relationships within the family Lucanidae and outgroups. Numbers next to each node represent Bayesian posterior probabilities (first number) and partitioned maximum likelihood bootstrap support (second number). This maximum clade credibility tree is based on MrBayes analysis of combined DNA sequence data from the mitochondrial 16S rDNA, the nuclear 18S rDNA, 28S rDNA, and the protein-coding gene WNG. The subfamilies and tribes recovered as non-monophyletic groups are marked with asterisk. Images of exemplars are not to scale with scale bar next to each image represents 5 mm.

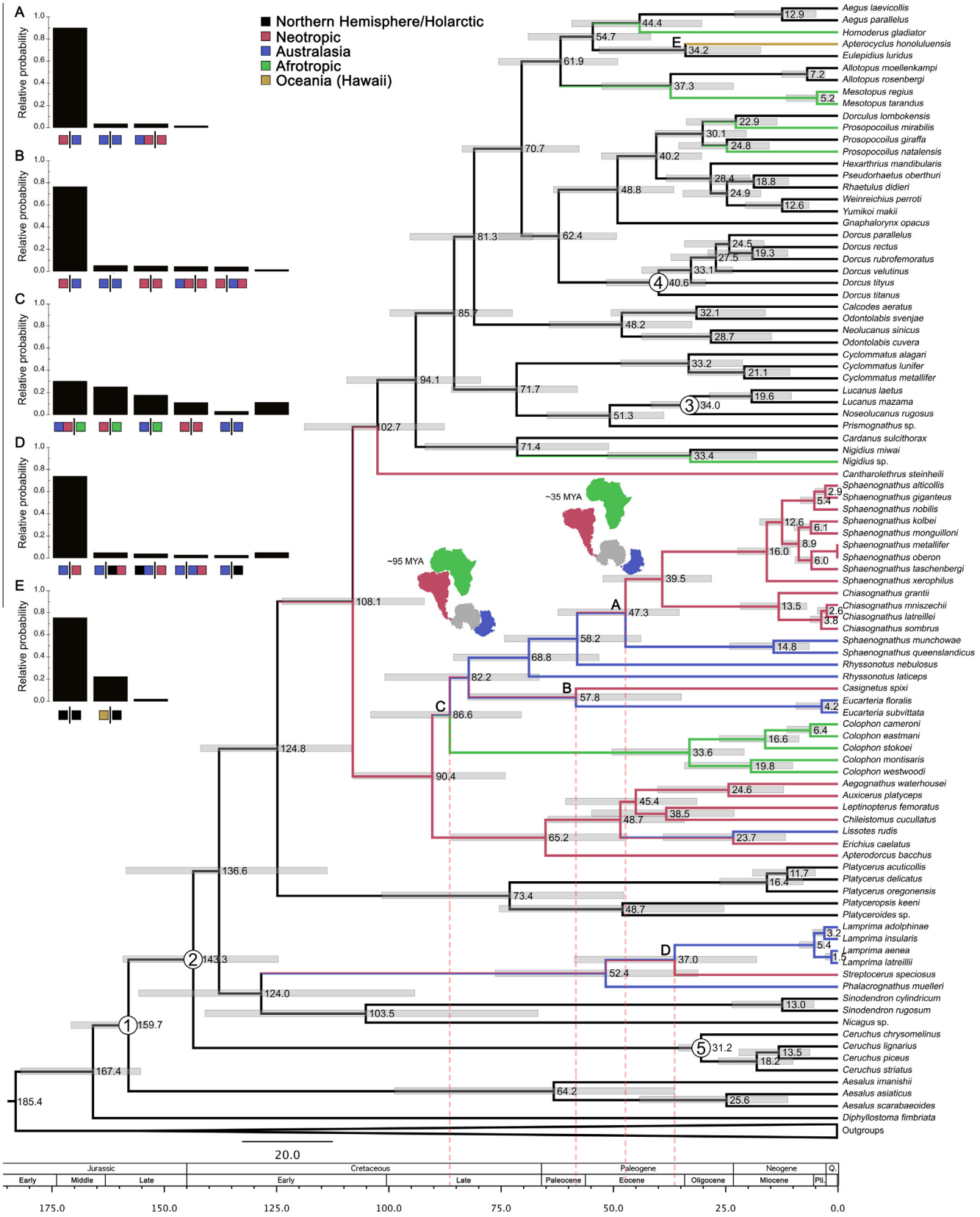

Fig. 2. Maximum clade credibility timetree obtained from BEAST based on five fossil calibrations and biogeographic reconstructions under the constrained maximum likelihood model for Lucanidae. Divergence time estimates are represented next to the nodes (in millions of years) with horizontal bars indicating 95% highest posterior density intervals. All branches are colored based on ancestral distributions inferred from Lagrange under the five-area model: Northern Hemisphere/Holarctic (black), Neotropical (red), Australasian (blue), Afrotropical (green), and Oceania (yellow). The five bar plots depict the likeliest scenarios (up to five) of ancestral distributions based on their relative probabilities at the corresponding node. Fossils used at five corresponding nodes are marked with numbers: (1) *Juraesalus atavus*; (2) *Prosinodendron krelli*; (3) *Lucanus fossilis*; (4) *Dorcus primigenius*; and (5) *Ceruchus fuchsii*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

50% had been collapsed. The Lucanidae *sensu lato* (i.e., Lucanidae + Diphyllostomatidae) was recovered as a monophyletic group with strong nodal support under both BI and MLI (1.0 BPP, 88% MLB), but the monophyly of the Lucanidae *s. s.* was weakly supported (<0.5 BPP, <50% MLB).

Within the Lucanidae *s. s.* clade, the subfamilies Lucaninae and Lampriminae were monophyletic with strong nodal support (1.0 BPP, 98% MLB for Lucaninae; 1.0 BPP, 100% MLB for Lampriminae). Other two subfamilies, the Aesalinae and Syndesinae, were paraphyletic and polyphyletic, respectively. While all three species of *Aesalus* formed a monophyletic group (1.0 BPP, 100% MLB), the other aesaline genus, *Nicacus*, formed a clade with a syndesine genus *Sinodendron* with weak nodal support (0.69 BPP, <50% MLB). Moreover, another syndesine genus *Ceruchus* was sister group to a clade that includes all members of the Lampriminae; however, the node including the *Ceruchus* and lamprimines was only weakly supported (0.63 BPP, <50% MLB). Within the Lampriminae, the tribe Lamprimini was polyphyletic, where the monotypic tribe Streptocerini appeared to be sister group to all *Lamprima* species (0.97 BPP, 95% MLB), with the genus *Phalacrognathus* of Lamprimini falling outside the *Lamprima-Streptocerus* clade.

Within the subfamily Lucaninae, 11 of the 18 tribes analyzed in the present study were found to be monophyletic with strong nodal support values. The tribes Dorcini, Lissapterini. Lucanini, Neoprosopocoilini, and Sclerostomini were recovered as polyphyletic, and the Cladognathini and Rhyssonotini as paraphyletic. Based on this topology, the Lucaninae could be further grouped into three distinct lineages. The earliest branching lineage was comprised solely of the tribe Platycerini whose monophyly was strongly supported under both BI and MLI (1.0 BPP, 100% MLB). Among the more derived lucanines, the second lineage was identified consisting of predominantly austral stag beetles, including the Chiasognathini, Rhyssonotini, Pholidotini, Colophonini, Neoprosopocoilini (*Apterodorcus* + *Aegognathus* + *Auxicerus* + *Leptinopterus*), Sclerostomini (*Erichius* + *Chileistomus*) and Lissapterini (*Lissotes*), whose monophyly was strongly supported (1.0 BPP, 86% MLB). The third lineage was composed of primarily Holarctic or rather cosmopolitan species, with the exception of the Neotropical genus *Cantharolethrus*. The monophyly of the third lineage was weakly supported under BI and moderately under MLI (0.48 BPP, 60% MLB), but the clade within this lineage without *Cantharolethrus* showed strong or moderate nodal support (1.0 BPP, 69% MLB).

3.2. Time of divergence

The estimation of divergence time using five fossil calibration points suggested that the crown group Lucanidae *s. s.* originated during the Late Jurassic circa 160 MYA (Fig. 2; 95% highest posterior density (HPD) confidence interval: 154–171 MYA). The stem group Lucanidae *s. s.* (i.e., the crown group Lucanidae *s. l.*, including Lucanidae *s. s.*, Diphyllostomatidae, and †Paralucanidae) arose during the Middle Jurassic circa 167 MYA (95% HPD: 155–182 MYA), while the stem group Lucanidae *s. l.* diverged from other primitive scarabaeoids during the Early Jurassic circa 185 MYA (95% HPD: 156–217 MYA). The stem group Aesalinae branched off from the rest of Lucanidae in the Late Jurassic, which is also supported by the fossil records of Jurassic and Cretaceous aesalines (Table 3). The splits among lamprimines first occurred between the monotypic genus *Phalacrognathus* and the *Lamprima-Streptocerus* clade during the early Eocene circa 52 MYA (95% HPD: 31–76 MYA). Then, the divergence of the Neotropical *Streptocerus* and the Australasian *Lamprima* was estimated to have occurred in the late Eocene circa 37 MYA (node D; 95% HPD: 18–59 MYA). Within the Lucaninae, the most primitive Platycerini lineage branched off first during the mid-Early Cretaceous around 125 MYA (95% HPD: 108–142 MYA), and the other lineage further diverged into the two main lineages at the end of Lower Cretaceous circa 108 MYA (95% HPD: 124–92 MYA), which then diversified rapidly in each hemisphere. Within the austral lineage, the only African genus *Colophon* seems to have diverged from the Neotropical-Australasian clade that includes the *Chiasognathus*, *Sphaenognathus*, *Rhyssonotus*, *Casignetus*, and *Eucarteria* during the mid-Upper Cretaceous circa 87 MYA (node C; 95% HPD: 70–104 MYA). The subsequent divergences within this Neotropical-Australasian clade led to the separations between the *Casignetus* and *Eucarteria* around 58 MYA (node B; 95% HPD: 35–83 MYA) and within the Chiasognathini around 47 MYA (node A; 95% HPD: 35–62 MYA). Although the divergence estimates at most nodes showed relatively narrow 95% HPD interval, the two most basal nodes that include the Lampriminae, *Sinodendron* and *Nicagus*, as well as the nodes among the three *Aesalus* species, showed exceptionally wide 95% HPD intervals, suggesting that fossil calibrations had substantial influence on divergence estimates at these nodes.

3.3. Biogeographic analyses

Under our five-area biogeographic model, it is hard to accurately interpret the origin of the Lucanidae. Yet, our analysis favored the Northern Hemisphere origin of the Lucanidae with high relative probability (*relative prob.* = 0.803; $\ln L = -94.94$), which was followed by two subsequent, independent dispersal events to the Southern Hemisphere between the Lower Cretaceous and early Paleogene. The first major dispersal event occurred among the ancestors of present-day Lampriminae, which would have migrated into Gondwana between 52.4 MYA and 124 MYA. Within the Lampriminae, the divergence between *Lamprima* and *Streptocerus* was explained by a vicariance event between the Australasian and the Neotropics (*relative prob.* = 0.786; $\ln L = -94.96$). The second main dispersal event happened in the Lower Cretaceous among the ancestors of the modern Lucaninae, some of which have diversified into a lineage whose current disjunction distribution was resulted by the sequential break-up of Gondwana. The relative probability of the most likely dispersal scenario inferred under the DEC model was plotted at each internal node (Fig. S3). A Neotropical origin of the clade including the Chiasognathini, Colophonini, Pholidotini, and Rhyssonotini was inferred, of which the first divergence occurred to the Colophonini of South Africa through a vicariance event between the Neotropical-Australasian block and the Afrotropics (*relative prob.* = 0.305; $\ln L = -95.91$; Fig. 2c). The second most likely scenario suggested a vicariance event between the Neotropics and the Afrotropics with comparably high relative probability (*relative prob.* = 0.252; $\ln L = -96.1$). Though we could not determine one specific scenario for this node, it is clear that the divergence of Colophonini represents a vicariance event associated with the separation of Africa from the rest of Gondwana. The Chiasognathini was inferred to have originated in the Australasian region (*relative prob.* = 0.609; $\ln L = -95.22$), but the Gondwanan origin across the present-day Neotropical and Australasian regions could not be completed ruled out (*relative prob.* = 0.374; $\ln L = -95.7$). However, a vicariance explanation for the divergence between the Australasian and the Neotropical Chiasognathini was strongly supported (*relative prob.* = 0.902; $\ln L = -94.82$; Fig. 2a). An additional grouping that showed the Gondwanan relationship was *Casignetus* and *Eucarteria*. The ancestors of the two genera would have originated in Gondwana across the present-day Neotropical and Australasian regions (*relative prob.* = 0.422; $\ln L = -95.58$), and the divergence between the two lineages clearly represents a vi cariance event (*relative*

prob. = 0.762; $\ln L = -94.99$; Fig. 2b). Lastly, the Northern Hemisphere/Holarctic origin of the Hawaiian lucanid genus *Apterocyclus* was suggested (*relative prob.* = 0.757; $\ln L = -95.0$; Fig. 2e), which, in turn, indicates that some lineages of the *Apterocyclus-Eulepidius* ancestors would have dispersed presumably from the Indomalaya or Palearctic region across the ocean and colonized Kaua'i of the Hawaiian Islands.

4. Discussion

4.1. Phylogenetic accounts

This study presents the first comprehensive molecular phylogeny of world Lucanidae. The combination of mitochondrial rDNA (16S), nuclear rDNA (18S and 28S), and NPC (WNG) genes represents the evolutionary history of Lucanidae at a wide range of molecular evolutionary rates, which allowed for excellent resolution of lucanid relationships. Overall, the phylogeny estimate suggested that the taxa included in this study well represent the world lucanid diversity, and there was no apparent effect of long-branch attraction (LBA) observed within the ingroup. In general, the basal relationships appeared to be less thoroughly resolved, particularly under MLI, which may be an artifact of using short, partial sequences of nuclear rDNA, or insufficient sampling among early branching lineages (e.g., Aesalinae: Ceratognathini and Syndesinae: Syndesini).

Morphologically, the Lucanidae *s. s.* is clearly a monophyletic group, distinct from the Diphyllostomatidae, but the nodal support for this clade was weak. On the other hand, the monophyly of the Lucanidae *s. l.* clade that includes Diphyllostomaidae was strongly supported, suggesting that Diphyllostomatidae is not merely a sister group to Lucanidae, but represents an ancient lineage that had branched off first from the crown group Lucanidae. As a monotypic family with only three species all described from California, Diphyllostomatidae can be considered a primitive lucanid lineage that has retained its ancestral states for over 160 million years.

The problems with current lucanid classification are clear in our phylogeny, indicating a need for major revisions of the current classification system. Several taxonomic changes are proposed, but left unformalized in this paper. At the level of subfamily, the Aesalinae and Syndesinae should be re-defined, as their monophyly has been contested. Nevertheless, our analyses lack major lineages in each of the two subfamilies, namely the Ceratognathini and Syndesini, respectively, and therefore, more comprehensive sampling must be done within these lineages for more accurate reconstruction of basal group relationships. Based on morphology, however, it is possible to separate out an aesaline clade that consists of the genera *Aesalus*, *Echinoaesalus*, *Lucanobium*, and *Trogellus* as a distinct group. All of these four genera exhibit unique male aedeagal structure with cylindrical and curved median lobe, to which extremely reduced parameres and basal piece are fused, and without eversible internal sac and struts. These characters clearly deviate from the conventional aedeagal shapes of lucanids, and are believed to be synapomorphies for this clade. A recent molecular phylogeny of Aesalinae based on 18S and 28 sequences, in fact, corroborates this view (Paulsen, 2013). However, our results of phylogenetic positions of an aesaline tribe Nicagini and a syndesine tribe Sinodendrini disagree with those of Paulsen (2013), in which the Nicagini formed a clade with the Ceratognathini and the Sinodendrini with lamprimines. Unfortunately, the sequence data for the species analyzed in Paulsen (2013) have not yet been made available on any public database, and therefore we could not include them in the present analyses. The phylogenetic position of the Nicagini-Sinodendrini clade in relation to the Ceruchini and Lampriminae differed even between our two Bayesian trees, one from MrBayes (Fig. 1) and the other from BEAST (Fig. 2). Nevertheless, the node connecting the clade, which includes Nicagini, Sinodendrini and Lampriminae, to the Lucaninae clade in a BEAST tree had almost no support (0.26 BPP) and thus this basal relationship in our BEAST phylogeny must be viewed as polytomy. In addition, the Lampriminae was recovered as a monophyletic group with strong nodal support, but the tribe Lamprimini appeared paraphyletic. Despite the current taxonomic position of *Lamprima* with another Australasian genus *Phalacrognathus* in the tribe Lamprimini, *Lamprima* rather formed a clade with a Neotropical tribe Streptocerini. Therefore, a separate tribal name should be given to *Phalacrognathus*.

Within the Lucaninae, the monophyly of most tribes and genera was strongly supported with seven out of the 18 tribes analyzed in this study appearing either polyphyletic or paraphyletic. The Neoprosopocoilini was the most problematic group whose members were placed in four different positions throughout the phylogeny. When Maes (1992b) described this tribe, it was mostly the distributional evidence that led to such grouping (Maes, personal communication). The Neoprosopocoilini is, in fact, one of the tribes whose names are considered invalid under the International Code of Zoological Nomenclature (i.e., nomen nudum), because it was not accompanied by a description or definition and its name was not based on an available generic name (Smith, 2006). Therefore, the genera currently classified under the Neoprosopocoilini require careful re-evaluation to establish more natural classification system, which may involve description of several new tribes.

In particular, the phylogenetic position of the mysterious Hawaiian genus *Apterocyclus* was revealed for the first time, which also provided insights into the geographical origin of the only native species of Scarabaeoidea in the Hawaiian Islands. *Apterocyclus* was recovered most closely related to *Eulepidius* Westwood from Borneo. Our biogeographic analysis suggests that the divergence between the two genera must have happened through dispersal, which is in agreement with the geographic history of the Hawaiian Islands. Taxonomically, *Apterocyclus* is currently placed within the tribe Neoprosopocoilini based on its superficial morphological similarities with a Neotropical genus *Apterodorcus*. On the other hand, *Eulepidius* belongs to the tribe Dorcini, presumably based on its morphological affinities with *Gnaphaloryx* and *Dorcus* (*Velutinodorcus*) of the same tribe. Nevertheless, the *Apterocyclus-Eulepidius* clade was recovered as a completely distinct lineage that is sister to the Aegini-Homoderini clade, so it certainly deserves its own tribal status.

Apart from the issues of classification, the phylogenetic position of Colophonini was also revealed for the first time in the present study. As Jeannel's hypothesis predicted, its potential common ancestry with the Chiasognathini is confirmed: The Colophonini was recovered as a sister group to an austral stag beetle clade that consists of the Chiasognathini, Rhyssonotini, and Pholidotini. This result conforms to Holloway's (1960) placement of the Colophonini in the Lucaninae based on the similarities in male aedeagal structures, and rejects its potential position in the Lampriminae as proposed by Didier and Séguy (1953). In fact, the close affinity of Colophonini with Chiasognathini is also supported by the feeding habit of the larvae. Unlike typical lucanid larvae which feed on rotten wood, the larvae of Chiasognathini and Colophonini have been reported to ingest humus-rich soil (Moore and Monteith, 2004; Onore, 1994; Scholtz and Endrödy-Younga, 1994).

On a more taxonomic note, we suggest transferring *Gnaphalorynx* and *Dorculus* to the tribe Cladognathini, and *Eucarteria* to the Pholidotini. Reid (1999) proposed synonymizing *Eucarteria* with *Cacostomus* and suggested the Neotropical genus

Casignetus to be its sister group. Although we tentatively retained *Eucarteria* as a valid genus, our phylogeny supported his view that *Eucarteria* is more closely related to *Casignetus* than to *Rhyssonotus*. Moreover, although we included only two species of *Rhyssonotus*, this genus was recovered as a paraphyletic group. Holloway (1960) observed several morphological characters, such as the number of antennal club segments, that differentiate *Rhyssonotus nebulosus* (Kirby) from other *Rhyssonotus* species. In fact, the number of antennal club segments is a relatively static morphological character in Lucanidae that rarely varies within a genus. Hence, some morphological evidence appears to partially support the paraphyly of *Rhyssonotus* inferred by our molecular analyses. Finally, the positions of the remaining Neoprosopocoilini, Lissapterini, and Sclerostomini remain untouched in the present paper, but could be established through more thorough sampling of related Neotropical genera and their potential "missing links," such as *Macrocrates*, in a more comprehensive phylogenetic study.

4.2. The ancestral lucanid

Lucanidae has long been considered as one of the most primitive groups of living scarabaeoids (Crowson, 1967; Howden, 1982; Iablokoff-Khnzorian, 1977; Lawrence and Newton, 1995). Based on fossil evidence of *Paralucanus mesozoicus* (Paralucanidae), Krell (2006) suggested Lucanidae to have diverged from its common ancestor with Passalidae during the Late Jurassic, for which he followed the phylogenetic system of Browne and Scholtz (1999, 1998). Although various phylogenetic analyses suggested Pleocomidae LeConte (Smith et al., 2006), Passalidae (Grebennikov and Scholtz, 2004) and Glaresidae (Scholtz et al., 1994) as the earliest branching lineages, a recent, comprehensive phylogeny of Scarabaeoidea showed Lucanidae and Glaresidae to be the oldest lineages (Ahrens et al., 2014).

We estimated the stem group lucanids to have arisen during the Middle Jurassic around 167 MYA (95% HPD: 155–182 MYA), and subsequently, the crown group lucanids during the Late Jurassic around 160 MYA (95% HPD: 154–171 MYA). Ahrens et al. (2014) estimated the crown group divergence time of Lucanidae to range from 91 to 147 MYA depending on the placement of the calibration point (i.e., either at the crown group or stem group node). The authors tentatively accepted 91 MYA as the age of crown group Lucanidae, which resulted from calibration at the stem group node. Although this age might appear much more recent than our divergence time estimate at 160 MYA, it, in fact, perfectly conforms to our result. The two lucanids included in their analyses belong to the genera *Lucanus* and *Nigidius*, respectively, whose divergence age is estimated to be around 94 MYA (95% HPD: 79–109 MYA) in our analyses. The two genera do not represent the earliest split within Lucanidae and hence, the dating analysis based on them would result in much recent divergence estimate.

More importantly, our results suggest that the Diphyllostomatidae is the sister group of all lucanids, and with its six visible sternites and exposed protrochantin, may represent the ancient lucanid form (Fig. 3). In fact, exposed protrochantin is considered the primitive morphological character in the Scarabaeoidea (Scholtz et al., 1994). Within the Lucanidae *s. s.*, *Aesalus* constituted the earliest branching clade, followed by the lamprimines and some syndesines (*Sinodendron* + *Nicagus*). As Handlirsch (1924) predicted, the lamprimines seem to have retained the ancestral lucanid characters, such as the 3-segmented antennal club and subcontiguous procoxae. Moreover, the syndesines exhibit straight or subgeniculate antennae in addition to aforementioned characters, all of which are characteristic of the Diphyllostomatidae (Fig. 3). An extinct family within the Lucanidae *s. l.*, Paralucanidae, from the Late Jurassic, in fact, shows six visible sternites, which seems to be a plesiomorphic character of stem group lucanids. Other Mesozoic lucanids, *Juraesalus atavus* and *Prosinodendron krelli*, generally have similar morphological plans, though both species more accurately fit the definition of crown group lucanids for having only five visible sternites.

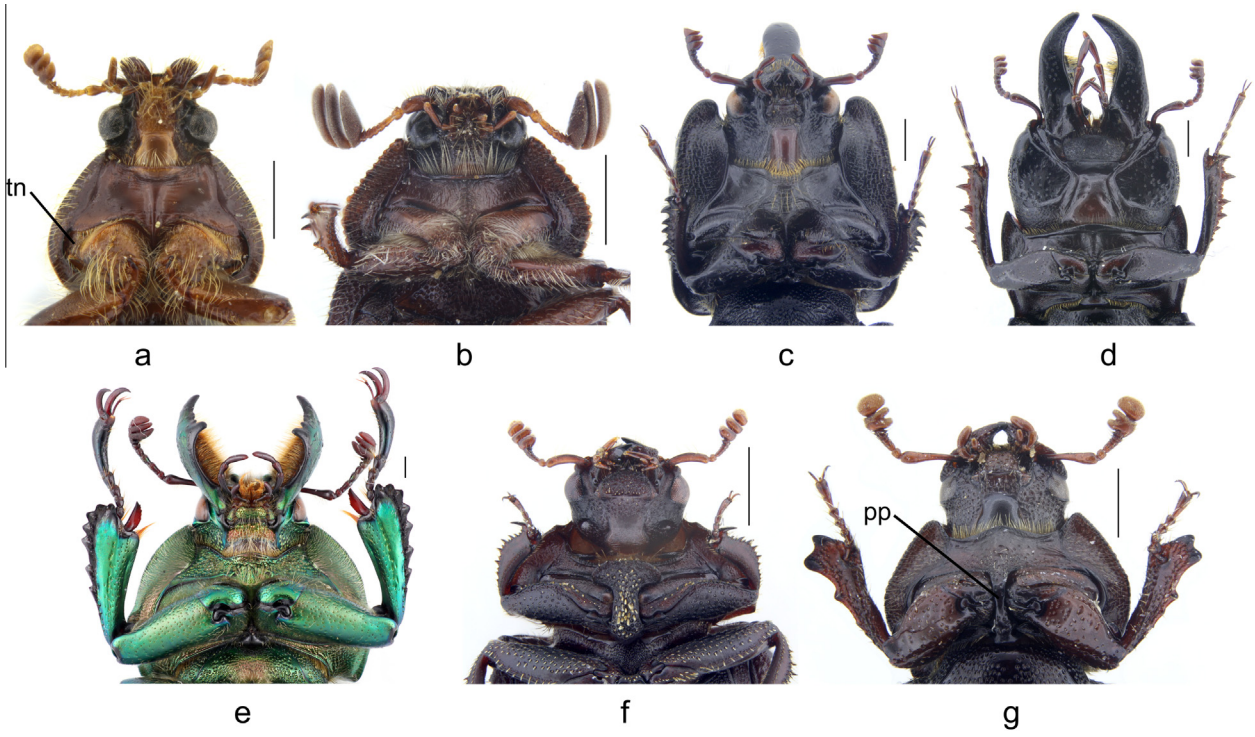

Fig. 3. Ventral view of the head and thorax in males showing the primitive lucanid characters in antennal structure, protrochantin (tn), procoxae, and prosternal process (pp): (a) *Diphyllostoma* (Diphyllostomatidae); (b) *Nicagus* (Lucanidae); (c) *Sinodendron*; (d) *Ceruchus*; (e) *Lamprima*; (f) *Aesalus*; and (g) *Platyceropsis*. Scale bar next to each image represents 1 mm.

Without conducting a phylogenetic analysis using morphological data or reconstructing ancestral character state on a currently available phylogeny, it is hard to accurately extrapolate the morphology of ancestral lucanids. Nevertheless, our results enable us to hypothesize that the extant Diphyllostomatidae and the extinct Paralucanidae well represent the stem group lucanids, which, having diverged from other primitive scarabaeoids during the Early Jurassic, gave rise to the present lucanid form in the Middle Jurassic.

4.3. The origin and evolution of sexually dimorphic mandibles

Evolutionary biologists have long been intrigued by spectacular evolution of complex morphological structures (Darwin, 1859; Mayr, 1953). Stunning structural innovation of beetle horns even led Darwin (1871) to describe the idea of sexual selection based on such traits. In *The Descent of Man*, Darwin (1871) wrote: "the mandibles of the common stag-beetle, and probably of many other species, are used as efficient weapons for fighting . . . We have seen that with the *Lucanus elaphus* of N. America they are used for seizing the female" (pp. 376–377). In fact, entomologists who studied stag beetles have frequently attempted to explain the evolutionary origin of exaggerated mandibles in males. Arrow (1937) recorded extreme variations in stag beetle mandibles even within a single species, and later concluded that lucanid mandibles, as well as other forms of scarab horns, must have evolved many different times independently (Arrow, 1951). Hosoya and Araya (2005) further supported these presumed multiple origins of mandible evolution in stag beetles based on the molecular phylogeny of Japanese stag beetles. In fact, Emlen et al. (2007) suggested that scarab beetle horns, including the mandibles of stag beetles, are structures that are relatively easy to gain or lose. Their study showed that emergence, reduction, and re-emergence of scarab horns involve extremely simple developmental mechanisms, and therefore, multiple origins of mandible evolution is very likely to occur. Our results corroborate this view that sexual dimorphism and exceptional development of male mandibles happened multiple independent times throughout the evolutionary history of stag beetles, followed by subsequent loss in some lineages that are nested within otherwise sexually dimorphic clades. There seems to have been two major independent origins of prolific mandible evolution: One in the Lampriminae and the other in a more derived lineage of Lucaninae excluding the Platycerini. One additional origin of weak dimorphism might have happened in a syndesine tribe Ceruchini. The Syndesinae was recovered as a polyphyletic group, but if the tribe Syndesini, which is absent in this study, forms a monophyletic group with the Ceruchini, this origin of weak dimorphism may apply to the Ceruchini-Syndesini group. The last tribe in the Syndesinae is Sinodendrini, which has developed its unique cephalic horn in males. We believe that this cephalic horn would provide the same functional advantage during male-male combat as those commonly found in the Dynastinae (Scarabaeidae).

Within the Lucaninae, most species exhibit a high degree of sexual dimorphism with a few notable exceptions: the Figulini, Nigidiini, *Colophon* (except *Colophon primosi* Barnard), *Apterocyclus*, and *Dorculus*. The most parsimonious explanation for these lucanine lineages would be that they have lost their dimorphic states secondarily. The Figulini and Nigidiini are closely related groups that are adapted to burrowing life style inside the decaying wood. In fact, these species do not engage in combat between rival males and are often found in subsocial colonies inside the highly limited space. Hence, negative selection against retaining costly male mandibles would have resulted in secondary loss of such a trait. In addition, *Colophon* and *Apterocyclus* are completely flightless stag beetles whose distributions are restricted to high altitude mountains of the Western Cape in South Africa, and the Island of Kaua'i among the Hawaiian Islands, respectively. We observed the common reduction of male mandibles among flightless genera (e.g., *Apterodorcus*, *Altitatiayus*, *Erichius*, *Geodorcus*, *Lissapterus*, *Lissotes*, and *Paralissotes*), and in case of *Colophon* and *Apterocyclus*, their subterranean habit might have caused a similar reduction of sexual dimorphism. The adults of the two genera are usually observed dwelling on the ground, rather than crawling on the trunk of a tree (Endrödy-Younga, 1988; Van Dyke, 1922). Because the male mandibles in stag beetles are usually used in conspecific combat to throw each other off the tree, such exaggerated structures would be incompetent and even cumbersome for subterranean life style, in which male-male combat must be accomplished in different forms.

4.4. Jeannel's hypothesis: Historical biogeography of the austral lucanids

The continental break-up of Gondwana has often been believed to have led to vicariance in the austral biotas. Among beetles, the Gondwanan relationships had been often noticed in adephagan beetles, such as the Carabidae Latreille and Dytiscidae Leach (e.g., Darlington, 1965; Erichson, 1842; Jeannel, 1942). As a relatively recent lineage, stag beetles have rarely been discussed in biogeographic context. The only group discussed to date is the 'Chiasognathinae,' which often refers to the austral stag beetles whose classification has changed drastically over the past century. Before beginning our discussion on Jeannel's hypothesis, it is important to redefine the 'Chiasognathinae.'

First described as a family by Burmeister (1847), Chiasognathidae has been treated variously as either a family (e.g., Burmeister, 1847; Parry, 1864; van Roon, 1910), subfamily (e.g., Brink, 1956; Chalumeau and Brochier, 2007, 2001; Didier and Séguy, 1953; Maes, 1992b) or tribe (e.g., Holloway, 1960; Krajick, 2001; Landin, 1955; Paulsen and Smith, 2010) for over a century. Holloway (1960), in her extraordinary discussion on interrelations of lucanid genera, reviewed the classification of the 'Chiasognathinae,' and suggested the affinities of *Chiasognathus*, *Sphenognathus* [sic], *Pholidotus* (=*Casignetus*), *Colophon*, and *Ryssonotus* [sic] with the typical lucanines, as opposed to with the lamprimines. It is now clear, based on male aedeagal characters (Holloway, 1960; Sharp and Muir, 1912), as well as on molecular evidence in the present study, that the Chiasognathini is a group of true lucanines, which clearly differs from lamprimines, and that its definition must be restricted to include only *Chiasognathus* and *Sphaenognathus*. Moreover, *Colophon* has also been classified under various subfamilies or tribes, but this group is evidently a true lucanine that merits establishment of a tribe, the Colophonini. Although much discussion on Jeannel's hypothesis (e.g., Chalumeau and Brochier, 2007, 2001; Moore and Monteith, 2004; Moore, 1978) has been limited to Chiasognathini, it is noteworthy that Jeannel's original hypothesis applies to six tribes of two subfamilies under the current classification scheme. As a first attempt to evaluate Jeannel's hypothesis, our discussion treats all six tribes.

As Jeannel's hypothesis predicted, the Chiasognathini clearly exhibits the Gondwanan distribution between the Neotropical and Australasian regions. Although the distribution of the genus *Chiasognathus* is restricted to the Chilean Andes with few species extending to Argentina, the genus *Sphaenognathus* consists of about 30 species, two of which are found in tablelands of Northeastern Australia and all the others occurring at high altitudes along the Andes Mountains as far north as Colombia and Venezuela. Our phylogeny and biogeographic analysis under the DEC model suggests a close relationship between the species of the two continents, which diverged circa 47 MYA (95% HPD: 35–62 MYA; Fig. 2) through a vicariance event caused by the complete separation of Australia from the South America-Antarctica block in

the late Eocene (35 MYA) (Sanmartín and Ronquist, 2004). Our biogeographic analysis recovered the ancient distribution of the ancestral species of Chiasognathini to be restricted to either the present-day Australasian region of Gondwana or a slightly wider area across the modern Australasian and Neotropical regions. In case of the former scenario, the ancestral species of Chiasognathini would have expanded its distribution across Gondwana from mainly the modern Australasian into the Neotropics through Antarctica. Nevertheless, based on our biogeographic analysis, a vicariance explanation could clearly account for the divergence between the Australasian and Neotropical Chiasognathini.

In addition, the genus *Colophon*, of which the distribution is restricted to the high mountain peaks of the Western Cape, South Africa, seems to have branched off from the austral stag beetle clade that consisted of the Chiasognathini, Rhyssonotini, and Pholidotini circa 87 MYA (95% HPD: 70–104 MYA). Though this divergence barely preceded the time when Africa achieved its complete separation from South America in the mid-late Cretaceous (80–110 MYA) (Beaulieu et al., 2013), it is probable that the divergence of *Colophon* also represents an ancient Gondwanan relict. Our biogeographic analysis indicates that the ancestor of *Colophon* and other aforementioned austral lineages have originated in the present-day Neotropical region of Gondwana. This ancestral lineage seems to have flourished during the mid-late Cretaceous across the entire region of a "Southern Temperate Gondwana" province, which consisted of southern South America, southern Africa, Australia, Antarctica, New Caledonia, and New Zealand (Sanmartín and Ronquist, 2004). Following the break-up of the Afrotropics from Gondwana, this widespread ancestral lineage then diverged into two separate lineages through vicariance, one of which gave rise to *Colophon* in the Afrotropics and the other which remained widespread across the Australasian and Neotropical regions. In fact, our biogeographic scenario conforms to Endrödy-Younga's (1988) hypothesis on *Colophon*'s lowland origin, which was further corroborated by a recent molecular phylogenetic study of *Colophon* (Switala et al., 2014). Based on this information, it can be hypothesized that modern high-altitude mountains in each continent would have served as refugia for once vagile ancient stag beetle lineages in the southern temperate Gondwana. Another example of the Gondwanan distribution within the aforementioned austral stag beetle clade was recovered between the Neotropical genus *Casignetus* and the Australasian genus *Eucarteria*. As Reid (1999) correctly predicted the close affinity between these two genera, they seem to be sister groups that have diverged circa 58 MYA (95% HPD: 35–82 MYA).

The last group relevant to Jeannel's hypothesis is the subfamily Lampriminae. Stunning morphological similarities between the Australasian *Lamprima* and the Neotropical *Streptocerus* were confirmed in the recovered monophyly of this group that diverged circa 38 MYA (95% HPD: 31–76 MYA). This age is younger than the divergence observed in the Chiasognathini, and the 95% HPD confidence interval even extends younger than the age when Australia was separated from the South America-Antarctica block. Unlike the Chiasognathini whose habitat is restricted to temperate highland regions, the lamprimines are distributed along the lowland of Southeastern Australia, Lord Howe Island, Norfolk Island, New Zealand and New Guinea in the Australasian, as well as Southwestern Chile and a small adjacent Argentinian region in the Neotropics. The presence of lamprimines on the Australian islands and New Zealand indicates their dispersal ability across the ocean. Though this may not suggest the possibility of trans-oceanic dispersal between the Australasian and the Neotropics across the Pacific Ocean, consistent gene flow might have persisted between the two continents even after the opening of the South Tasman Sea that separated Australia from the South America-Antarctica block around 35 MYA (Sanmartín and Ronquist, 2004). Furthermore, our biogeographic analysis predicted the distribution of the ancestral lineage of *Lamprima* and *Streptocerus* to be the present-day Australasian and Neotropical regions across Gondwana, and their divergence to be the result of vicariance. Therefore, a vicariance explanation based on the continental break-up still appears probable for the current disjunction pattern of lamprimines, even though their divergence between the two continents happened more recently than that of the Chiasognathini.

Overall, Jeannel's hypothesis concerning the biogeographic scenarios of austral stag beetles is correct. Our time-calibrated phylogeny and biogeographic scenarios under the DEC model bolstered Jeannel's argument that the Chiasognathini, Colophonini, Lamprimini, Pholidotini, Rhyssonotini, and Streptocerini had originated in Gondwana and diversified following the continental break-up pattern. In fact, the divergence history among these lineages closely corresponds to the sequential break-up of Gondwana. Although many entomologists, including Jeannel himself, have sought an explanation for the European fossil species of the Chiasognathini, it is dubious as to whether both *Palaeognathus succini* and *Protognathinus spielbergi* actually belong to this tribe. *Protognathinus spielbergi* most likely represents the lamprimines, but the taxonomic placement of *Paleognathus succini* remains unclear. *Palaeognathus succini* has been placed variously under the Lampriminae, Chiasognathini and even Cladognathini of the Lucaninae, and hence, an attempt to draw a link between the extant Chiasognathini and *P. succini* seems futile. Instead, Holdhaus' (1929) idea of a once worldwide distribution reduced to current distribution in the Southern Hemisphere perhaps applies more to the subfamily Lampriminae. As one of the primitive lineages of the Lucanidae, it is probable that the Lampriminae would have thrived in both hemispheres until the Eocene, thereby leaving a fossil of *P. spielbergi* in Europe.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.02.015.

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