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Phylogenetics and biogeography of the dung beetle genus *Onthophagus* inferred from mitochondrial genomes

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ABSTRACT

Phylogenetic relationships of dung beetles in the tribe Onthophagini, including the species-rich, cosmopolitan genus *Onthophagus*, were inferred using whole mitochondrial genomes. Data were generated by shotgun sequencing of mixed genomic DNA from >100 individuals on 50% of an Illumina MiSeq flow cell. Genome assembly of the mixed reads produced contigs of 74 (nearly) complete mitogenomes. The final dataset included representatives of *Onthophagus* from all biogeographic regions, closely related genera of Onthophagini, and the related tribes Onitini and Oniticellini. The analysis defined four major clades of Onthophagini, which was paraphyletic for Oniticellini, with Onitini as sister group to all others. Several (sub)genera considered as members of *Onthophagus* in the older literature formed separate deep lineages. All New World species of *Onthophagus* formed a monophyletic group, and the Australian taxa are confined to a single or two closely related clades, one of which forms the sister group of the New World species. Dating the tree by constraining the basal splits with existing calibrations of Scarabaeoidea suggests an origin of Onthophagini *sensu lato* in the Eocene and a rapid spread from an African ancestral stock into the Oriental region, and secondarily to Australia and the Americas at about 20–24 Mya. The successful assembly of mitogenomes and the well-supported tree obtained from these sequences demonstrates the power of shotgun sequencing from total genomic DNA of species pools as an efficient tool in genus-level phylogenetics.

1. Introduction

Dung beetles (Scarabaeinae) include ca. 6000 described species and are distributed across all continents except Antarctica (Simmons and Ridsdill-Smith, 2011b). Most species utilize animal faeces for feeding and breeding, which is a vital part of key ecological processes, including nutrient cycling and seed dispersal (Nichols et al., 2008). Within the Scarabaeinae, the genus *Onthophagus* Latreille 1802 constitutes a major portion of the known diversity, and with ca. 2300 species (Schoolmeesters, 2016) it is one of the most species-rich genera in the world (Roskov et al., 2013). *Onthophagus* is unusual among the genera of Scarabaeinae in its global distribution. The genus is considered a 'young' group in Cambefort's (1991) classification of dung beetle tribes and possibly diversified in the Cenozoic (around 23–33 million years ago) in parallel with the expanding grasslands and the radiation of mammals (Cambefort, 1991; Darlington, 1957; Davis et al., 2002; Emlen et al., 2005). This is broadly corroborated by a clock-calibrated tree of Scarabaeinae that finds the crown group of Onthophagini to be of fairly recent origin (Monaghan et al., 2007), although the study did not attempt an absolute-age estimation. Based on geographic distributions and ancestral area reconstructions, the genus possibly originated in the Afrotropical region (Emlen et al., 2005; Monaghan et al., 2007; Philips, 2016; Tarasov and Solodovnikov, 2011) and may have dispersed gradually to the Palearctic and Oriental regions followed by colonization of Australasia via south-east Asian islands and the New World from Afro-Eurasian origins (Davis et al., 2002). However, current phylogenetic hypotheses contradict this biogeographic scenario, and instead imply multiple long distance dispersal events (Monaghan et al., 2007).

Highest species diversity is seen in the Afrotropical and Oriental regions, which harbour as many as 1000 and 600 species,

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respectively (Tarasov and Solodovnikov, 2011). The high rate of speciation is thought to result from habitat isolation through specialization on novel dung sources and intense competition for the ephemeral food source (Davis and Sutton, 1997; Larsen et al., 2006; Simmons and Ridsdill-Smith, 2011a), in addition to sexual selection that drives the intriguing morphological diversification of head and thoracic horns used as weapons (Emlen et al., 2005).

Early classifications of the Scarabaeinae were based on the division between different types of dung handling, i.e. tunnelling and rolling (Balthasar, 1963; Janssens, 1949), for the two behavioural types that either build brood chambers directly beneath the dung pat or move the dung away by forming dung balls before burial. However, phylogenetic analyses did not confirm this split into tunnellers and rollers, and instead suggested the polyphyly of both behavioural types (Philips et al., 2004; Monaghan et al., 2007). Yet, the Onthophagini have been found to be part of a large lineage of tunnellers that includes the related tribes Onitini and Oniticellini. The attempts to clarify the phylogeny and classification of *Onthophagus* were hampered by the great species richness and by convergent evolution of morphological characters (Tarasov and Solodovnikov, 2011). Existing molecular phylogenetic analyses (Emlen et al., 2005; Mlambo et al., 2015; Monaghan et al., 2007; Villalba et al., 2002) included only a limited number of taxa and some of these analyses were restricted to specific geographic regions, while using only a few mitochondrial and nuclear loci. Owing to different choices of genes and taxon sampling that were largely non-overlapping there is little congruity among these phylogenetic studies (Tarasov and Solodovnikov, 2011).

To a large extent, these differences in approach by previous studies can be explained by the fact that clear diagnostic characters for subgeneric grouping have not been defined and most species have not been assigned to any higher-level taxa. Therefore, specific questions about the relationships of species and subgroups of *Onthophagus* are now arising from these initial studies. Also, initial phylogenetic conclusions are supported by various studies, such as: (1) the early split of lineages separating the (sub)genera *Proagoderus, Digitonthophagus* and *Phalops* from other lineages of Onthophagini (Monaghan et al., 2007; Philips, 2016; Tarasov and Solodovnikov, 2011); (2) the inclusion of *Caccobius, Cleptocaccobius* and *Milichus* within *Onthophagus* (Monaghan et al., 2007; Philips, 2016; Villalba et al., 2002); (3) support for distinct clades of *Onthophagus* confined to the New World and Australian regions (Emlen et al., 2005; Monaghan et al., 2007), and (4) the grouping of these clades with Oriental taxa in the '*Onthophagus propria*' (Tarasov and Solodovnikov, 2011).

In addition, existing studies have struggled to demonstrate the monophyly of Onthophagini with regard to the closely related tribes Oniticellini and Onitini. The Onthophagini were polyphyletic whenever a range of Oniticellini and Onitini were included (Mlambo et al., 2015; Monaghan et al., 2007; Ocampo and Hawks, 2006; Wirta et al., 2008). Other studies recovered monophyly (Vaz-de-Mello, 2007), but this may be an artefact due to limited taxon sampling outside of Onthophagini (Tarasov and Solodovnikov, 2011), while in another study monophyly was recovered with weak support values (Philips, 2016). Moreover, studies in the past have often tried to untangle the evolutionary history of the entire subfamily Scarabaeinae, rather than focusing on Onthophagini, and therefore sampled a low number of taxa per tribe with limited relevance to their internal relationships (see Scholtz et al., 2009; Tarasov and Génier, 2015).

The current study addresses the limited gene sampling and consequently weak branch support of existing analyses by using whole mitochondrial genomes (mitogenomes) for a representative set of samples of Onthophagini from each major biogeographic region and the closely related tribes Oniticellini and Onitini. Although phylogenetic analysis based solely on mitogenomes is often controversial, especially for inferring deep relationships (Carapelli et al., 2007; Hassanin et al., 2005; Masta et al., 2009), they have proven to be a powerful marker in phylogenetic studies at various hierarchical levels (e.g. Andújar et al., 2015; Bernt et al., 2013; Crampton-Platt et al., 2015; Gillett et al., 2014; Simon and Hadrys, 2013). Recent methods for shotgun sequencing and assembly from mixtures of species samples can greatly reduce the cost and effort of gathering mitogenomes, but the assembly may produce errors when close relatives are present in the mixture (Gómez-Rodríguez et al., 2015). Greater taxon sampling could improve phylogenetic inference from mitogenomes because of the improved modelling of character variation, which may overcome the widely acknowledged problems from rate heterogeneity and compositional heterogeneity that frequently confound phylogenetic inferences from mitochondrial DNA (e.g. Talavera and Vila, 2011). We show here that shotgun sequencing of pooled genomic DNAs from closely related species within the genus *Onthophagus* provides chimera-free assemblies of mitogenomes for most of the species in the pool, demonstrating the feasibility of phylogenetic shotgun mitogenomics also at this hierarchical level. The analysis resulted in well-supported relationships, both within *Onthophagus* and between the three closely related tribes Onthophagini, Oniticellini and Onitini. Linking the time of origin and diversification with distribution of these lineages we test biogeographical scenarios for the dispersal of Onthophagini around the world.

2. Material and methods

2.1. Taxon sampling

DNA extracts available at the Natural History Museum's Molecular Collections Facility were used for this study. Many of these individuals had already been included in previous studies, using partial mitochondrial *rrnL* and *cox1* genes and nuclear 28S rRNA genes (Inward, 2003; Monaghan et al., 2007). Additional DNA extractions were performed on ethanol-preserved specimen of species not present in the DNA collections using Qiagen tissue DNA extraction kits (supplementary table 1). Specimens included in the analysis were selected to represent a wide taxonomic and geographical coverage of the genus *Onthophagus* from the Afrotropical, Neotropical, Oriental and Palearctic regions, in addition to representatives of Onitini and Oniticellini. Outgroups for Illumina sequencing included *Coprophanaeus* (tribe Phanaeini) and *Eurysternus* (Eurysternini), and the mitogenome of a more distant outgroup (*Aphodius* sp. of the subfamily Aphodinae) was obtained from Timmermans et al. (2016). A total of 112 species were included: eight Onitini, 11 Oniticellini, 86 Onthophagini, six Eurysternini and a single species of Phanaeini (for a complete list of included specimens see supplementary table 1).

2.2. Sample pooling and Illumina sequencing

The concentration of double-stranded DNA for all extractions was determined with a Qubit dsDNA high-sensitivity kit (Invitrogen), and samples were pooled in equal concentrations to maximize assembly success. Two separate pools were established based on the presumed relatedness of species, separating close relatives to avoid chimera formation. TruSeq nano libraries (Illumina) were generated aiming for a large insert size to aid the assembly of mitogenomes (Crampton-Platt et al., 2015). The two libraries were sequenced in separate 600-cycle Illumina Miseq runs to obtain 2×300 bp paired-end reads (Illumina Inc., San Diego, CA).