Molecular phylogeny of the dung beetle fauna (Coleoptera: Scarabaeidae) of the Western Ghats biodiversity hotspot

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The tribal and generic-level phylogeny of Scarabaeinae (Coleoptera: Scarabaeidae) dung beetles have been often debated globally. However, fauna from India have not been a part of these analyses due to lack of data. We used partial sequences of 16S mtDNA gene of dung beetles collected from different parts of the Western Ghats, India, to examine (i) the tribal positions of Onthophagini, Onitini and Oniticellini, and (ii) the phylogenetic position of different genera of Onthophagini. We found that Oniticellini nested within Onthophagini, suggesting the invalid position of Oniticellini. The non-*Onthophagus* genera of Onthophagini – *Caccobius*, *Cleptocaccobius*, *Milichus* – nested within *Onthophagus*, suggesting that these three genera might be invalid and could be subgenera of *Onthophagus.* Onitini formed a separate clade in the phylogenetic tree. The results suggest for tribal-level reclassification of dung beetles, as noted in previous studies. The present study may enrich the molecular data of the Indian dung beetles, which are currently lacking.

Keywords: Biodiversity hotspot, dung beetles, molecular phylogeny.

THE dung beetles belonging to the sub-family Scarabaeinae are cosmopolitan, with 6775 species distributed worldwide[1]. They are important biodiversity indicator species with fascinating natural history[2–4]. On the global scale, there is only a little agreement on the biogeographic history of the evolution of dung beetles. For the cosmopolitan distribution of dung beetles, the biogeographic possibilities suggested are an out-of-Africa hypothesis[5–7] or Gondwanan vicariance and African dispersal for the dung beetle evolution and dispersal[8–10]. The nesting behaviour was relied upon for their tribal-level classification[11,12]. Balthasar[11,12] classified them into 12 tribes; of these, 6 were rollers and the other 6 were tunnellers. Until recently, 11 or 12 tribes have been considered valid[13,14]. Recent molecular studies were pivotal for reclassifying the dung beetle tribes. At present, 16 tribes have been considered valid[14–17]. They are Ateuchini, Byrrhidiini, Coprini, Deltochilini, Dichotomiini, Endroedyolini, Eucraniini, Gymnopleurini, Odontolomini, Oniticellini, Onitini, Onthophagini, Parachoriini, Phanaeini, Scarabaeini and Sisyphini[14–16].

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Onthophagini is the largest lineage that includes the tunnellers[18]. It is considered to be a modern tribe with a recent origin[18,19]. Oniticellini and Onitini are closely related tribes to Onthophagini[20,21]. The cosmopolitan genus *Onthophagus* falls under the tribe Onthophagini. *Onthophagus* is considered to be diversified around 23–33 million years ago in the Cenozoic age along with the diversification of mammals[7,22,23]. However, fossil records and molecular dating studies suggested that they were of Mesozoic origin[9,21,23]. It is suggested that *Onthophagus* have a possible Afro-tropical origin[3,22,24,25] and subsequent range expansions into other biogeographical zones[8,18]. The molecular phylogeny studies, however, suggested multiple dispersal events for *Onthophagus*[3,18].

Previous studies have suggested polyphyly for Onthophagini[3,20,21,26]. Some studies have suggested monophyly for this tribe[22,24,27,28]. However, the species in Oniticellini and Onitini used by Monaghan *et al.*[3] and Wirta *et al.*[21] were not used by Emlen *et al.*[22] in their study. Tarasov and Solodovnikov[25] performed a morphology-based phylogenetic analysis of the *Serrophorus* complex of Onthophagini. They used 91 morphological characters of 52 species and several genera of Onthophagini and related tribes. The results were congruent with those of Emlen *et al.*[22] and incongruent with those of Monaghan *et al.*[3] and Wirta *et al.*[21]. Monaghan *et al.*[3] had suggested that some basal lineages of the Onthophagini may belong to Onitini or Oniticellini. Breeschoten *et al.*[18] studied the tribal phylogeny by exclusively focusing on *Onthophagus* and found that all the New World Onthophagini formed a monophyletic group.

Most of the dung beetle phylogeny studies, either based on morphology or molecular traits, have lacked representative species from the Indian tropics. The lack of sequences from India in the publicly available databases might be a reason for taxonomic impediment. We aim to fill this critical gap through the present study. We used partial sequences of 16S (*rrnL*) gene of dung beetles collected from the Western Ghats biodiversity hotspot to understand the phylogeny of dung beetle tribes. We specifically examined (i) the tribal positions of Onthophagini, Onitini and Oniticellini of the Western Ghats biodiversity hotspot, and (ii) the position of different genera of Onthophagini. Mitochondrial genes are commonly used as reliable molecular markers to study the phylogeny and biogeography of closely and distantly related taxa among insects[29,30].

Dung beetles were sampled from various sites of the Western Ghats in the Indian peninsula – Kodagu (12°0.489′N, 76°2.279′E and 12°16.108′N, 75°38.592′E), Kasaragod (12°17.14′N, 75°15.1′E and 12°8.200′N, 75°9.384′E) and Thiruvananthapuram (8°17.512′N, 77°6.468′E and 8°47.686′N, 76°45.902′E). We used cow-dung pads for sampling beetles. The collected dung beetles were transferred to 99% ethanol immediately. They were identified to species level using the methods of Arrow[31] and Balthasar[11,12].

Table 1. Details of species used for phylogeny based on 16S (*rrnL*) in the study

GenBank accession no.	Tribe	Genus	Species	Author	Collected from	Region
KU739500	Onthophagini	*Onthophagus*	*longimanus*	Bates, 1887	Belize	Neotropic
KU739499	Onthophagini	*Onthophagus*	*nitidior*	Bates, 1887	Belize	Neotropic
KU739498	Onthophagini	*Onthophagus*	*rhinolophus*	Harold, 1869	Belize	Neotropic
KU739497	Onthophagini	*Digitonthophagus*	*gazella*	Frey, 1971	Madagascar	African
KU739491	Oniticellini	*Drepanocerus*	*kirbyi*	Kirby, 1828	South Africa	African
KU739490	Oniticellini	*Euoniticellus*	*intermedius*	Reiche, 1850	South Africa	African
KU739489	Oniticellini	*Helictopleurus*	*quadripunctatus*	Olivier, 1789	Madagascar	African
KU739488	Oniticellini	*Liatongus*	*militaris*	Laporte de Castelnau, 1840	South Africa	Australian
KU739487	Oniticellini	*Oniticellus*	*egregius*	Klug, 1855	South Africa	African
KU739486	Oniticellini	*Yvescambefortius*	*sarawacus*	Gillet, 1926	Indonesia	Oriental
KU739485	Oniticellini	*Tiniocellus*	*spinipes*	Roth, 1851	South Africa	African
KU739484	Onthophagini	*Caccobius*	*nigritulus*	Klug, 1855	South Africa	African
KU739436	Onthophagini	*Cleptocaccobius*	*convexifrons*	Raffray, 1877	South Africa	African
KU739481	Onthophagini	*Milichus*	*apicalis*	Fahraeus, 1857	South Africa	African
KU739480	Onthophagini	*Onthophagus*	Near *babirussa*	Indonesia	Oriental	
KU739478	Onthophagini	*Onthophagus*	*haematopus*	Harold, 1875	Ecuador	Neotropic
KU739477	Onthophagini	*Onthophagus*	*obscurior*	Boucomont, 1914	Indonesia	Oriental
KU739476	Onthophagini	*Onthophagus*	*rorarius*	Harold, 1877	Indonesia	Oriental
KU739475	Onthophagini	*Onthophagus*	sp.	–	South Africa	African
KU739474	Onthophagini	*Onthophagus*	*vulpes*	Harold, 1877	Indonesia	Oriental
KU739473	Onthophagini	*Phalops*	*ardea*	Klug, 1855	South Africa	African
KU739472	Onthophagini	*Onthophagus*	*bicallosus*	Klug, 1855	South Africa	African
KU739471	Onthophagini	*Onthophagus*	*schwaneri*	Vollenhoven, 1864	Indonesia	Oriental
KU739469	Onitini	*Bubas*	*bubalus*	Olivier, 1811	Spain	Palearctic
KU739468	Onitini	*Heteronitis*	*castelnaui*	Harold, 1862	South Africa	African
KU739467	Onitini	*Onitis*	*alexis*	Klug, 1835	South Africa	African
KU739466	Onitini	*Onitis*	*falcatus*	Wulfen, 1786	Hong Kong	Palearctic
KU739431	Onitini	*Onitis*	*fulgidus*	Klug, 1855	South Africa	African
KU739430	Onthophagini	*Onthophagus*	*orientalis*	Harold, 1868	Cambodia	Oriental
KU739464	Onthophagini	*Onthophagus*	*baolocensis*	Ochi and Kon, 2015	Cambodia	Oriental
KU739463	Onthophagini	*Onthophagus*	*yukae*	Masumoto *et al*., 2002	Cambodia	Oriental
KU739462	Onthophagini	*Onthophagus*	c.f. *taurinus*	White, 1844	Cambodia	Oriental
KU739461	Onthophagini	*Onthophagus*	*gracilipes*	Boucomont, 1914	Laos	Oriental
KU739460	Oniticellini	*Scaptodera*	*rhadamistus*	Fabricius, 1775	Laos	Oriental
KU739459	Onthophagini	*Digitonthophagus*	*bonasus*	Fabricius, 1775	Laos	Oriental
KU739426	Onthophagini	*Onthophagus*	c.f. *tragus*	Fabricius, 1792	Laos	Oriental
KU739457	Onthophagini	*Phalops*	*barbicornis*	Lansberge, 1883	–	African
KU739456	Onthophagini	*Onthophagus*	*ochreatus*	D'Orbigny, 1897	–	African
KU739424	Onthophagini	*Onthophagus*	*laticollis*	Klug, 1835	–	African
KU739454	Oniticellini	*Tragiscus*	*dimidiatus*	Klug, 1855	South Africa	African
KU739453	Oniticellini	*Euoniticellus*	*fulvus*	Goeze, 1777	Spain (?)	Palearctic
KU739450	Onitini	*Cheironitis*	*hoplosternus*	Harold, 1868	South Africa	African
KU739452	Onthophagini	*Onthophagus*	*fimetarius*	Roth, 1851	South Africa	African
MT913390	Onitini	*Onitis*	sp.	–	India	Oriental
MT903415	Onthophagini	*Onthophagus*	*andrewesi*	Arrow, 1931	India	Oriental
MT903965	Onthophagini	*Onthophagus*	*bifasciatus*	Fabricius, 1781	India	Oriental
MT904010	Onthophagini	*Onthophagus*	*bronzeus*	Arrow, 1907	India	Oriental
MT904286	Onthophagini	*Onthophagus*	*castetsi*	Lansberge, 1887	India	Oriental
MT904662	Onthophagini	*Onthophagus*	*cervus*	Fabricius, 1798	India	Oriental
MT904883	Onthophagini	*Onthophagus*	*dama*	Fabricius, 1798	India	Oriental
MT905022	Onthophagini	*Onthophagus*	*duporti*	Boucomont, 1914	India	Oriental
MT905025	Onthophagini	*Onthophagus*	*fasciatus*	Boucomont, 1914	India	Oriental
MT905072	Onthophagini	*Onthophagus*	*favrei*	Boucomont, 1914	India	Oriental
MT905073	Onthophagini	*Onthophagus*	*fuscopunctatus*	Fabricius, 1798	India	Oriental
MT907290	Onthophagini	*Onthophagus*	*griseosetosus*	Arrow, 1931	India	Oriental
MT913524	Onthophagini	*Onthophagus*	*laevigatus*	Fabricius, 1798	India	Oriental
MT907292	Onthophagini	*Onthophagus*	*madoqua*	Arrow, 1931	India	Oriental
MT907293	Onthophagini	*Onthophagus*	*malabarensis*	Boucomont, 1919	India	Oriental
MT907467	Onthophagini	*Onthophagus*	*negligens*	Walker, 1858	India	Oriental
MT907468	Onthophagini	*Onthophagus*	*orientalis*	Harold, 1868	India	Oriental
MT907469	Onthophagini	*Onthophagus*	*parvulus*	Fabricius, 1798	India	Oriental

(*Contd*)

Table 1. (*Contd*)

GenBank accession no.	Tribe	Genus	Species	Author	Collected from	Region
MT907472	Onthophagini	*Onthophagus*	*quadridentatus*	Fabricius, 1798	India	Oriental
MT907474	Onthophagini	*Onthophagus*	*rectecornutus*	Lansberge, 1883	India	Oriental
MT907499	Onthophagini	*Onthophagus*	*socialis*	Arrow, 1931	India	Oriental
MT908113	Onthophagini	*Onthophagus*	*spinifex*	Fabricius, 1781	India	Oriental
MT907514	Onthophagini	*Onthophagus*	*turbatus*	Walker, 1858	India	Oriental
MT908115	Onthophagini	*Onthophagus*	*unifasciatus*	Schaller, 1783	India	Oriental
MT908191	Onthophagini	*Onthophagus*	*usurpatus*	Balthasar, 1959	India	Oriental
MT908233	Onthophagini	*Onthophagus*	*vividus*	Arrow, 1907	India	Oriental
MT904654	Onthophagini	*Onthophagus*	*centricornis*	Fabricius, 1798	India	Oriental
MW362140	Aphodiini	*Aphodius*	sp.	–	India	Oriental
MW362138	Onthophagini	*Caccobius*	*aterrimus*	Fabricius, 1798	India	Oriental
MW348923	Oniticellini	*Tiniocellus*	*spinipes*	Roth, 1851	India	Oriental
MW348916	Oniticellini	*Oniticellus*	*cinctus*	Fabricius, 1775	India	Oriental
MW362139	Onthophagini	*Caccobius*	*meridionalis*	Boucomont, 1914	India	Oriental
EF656658	Aphodiini	*Aphodius*	sp.1	–	Madagascar	African
EF656659	Aphodiini	*Aphodius*	sp.2	–	Madagascar	African
EF656662	Aphodiini	*Aphodius*	sp.3	–	Madagascar	African
MW348573	Oniticellini	*Liatongus*	*indicus*	Arrow, 1908	India	Oriental

Voucher specimens were maintained and deposited in the entomology collection of the Central University of Kerala, Periya, India.

To ensure comprehensive geographical representation, sequences from various geographical regions, namely Afrotropical, Neotropical, Oriental and Palaearctic regions, were included in the analysis. They were downloaded from NCBI GenBank (www.ncbi.nlm.nih.gov). Representative species from Onitini and Oniticellini were also included in the analysis. *Aphodius* spp. of the tribe Aphodiini were designated as the outgroup for this study. A total of 79 species were included in the analysis as ingroup, which were 7 Onitini, 13 Oniticellini and 55 Onthophagini. Four species of *Aphodius* were used for rooting the tree as the outgroup.

A total of 38 species from the Western Ghats were included in the analysis: 1 Onitini, 3 Oniticellini, 28 Onthophagini and 1 Aphodiini. The genus *Onthophagus* included the maximum number (47) of species. Table 1 gives a detailed list of species included in the analysis, collection data and their GenBank accession number.

Genomic DNA was isolated from the thorax of small species and the hind leg of large species using QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Germany), following the instructions provided along with the kit with the following modifications: tissues were incubated overnight at 56°C in 180 µl of ATL buffer and 20 µl of proteinase K to completely lyse them. Isolated DNA was quantified and the quality was checked using a nanodrop spectrophotometer. It was further checked using the 100 bp Invitrogen ladder as control by agarose gel electrophoresis.

Approximately 520 bp of the 3′ end of 16S ribosomal RNA (*rrnL*) was amplified using the forward primer 16Sar 5′CGCCTGTTTAACAAAAACAT3′ (ref. 32) and reverse primer 16SB2 5′CTCCGGTTTGAACTCAGATCA3′ (ref. 3). Whenever amplification was not possible with 16SB2, another primer, 16Sb2 5′TTTAATCCAACATCGAGG3′ was used[3]. The PCR reaction mixture was set up for 27 µl with the following components: 12.5 µl PROMEGA master mix (2×), 10 pmol forward primer, 10 pmol reverse primer, 4 µl template DNA and 6.5 µl nuclease-free water. The following PCR conditions were applied: initial denaturation for 3 min at 95°C followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 43.2°–44.3°C for 40 sec and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. DNA was amplified in the Eppendorf master cycle Pro-S. The PCR products were analysed for their quality and quantity by 2% agarose gel electrophoresis and using Nanodrop. The amplified PCR products were then purified using Invitrogen's PureLink PCR purification kit, according to the manufacturer's instructions. Sequencing was performed on the ABI 3730 system using Big Dye Terminator v3.1 kit.

The sequences were compared with those available in the NCBI GenBank by BLAST search (https://blast.ncbi.nlm.nih.gov) to confirm the morphological identification. Species identity could be confirmed only up to the genus level for certain species, since the sequences of the species used were not available in the database. The quality of the sequences was checked using Sequence Scanner Software 2 v2.0 (Applied Biosystems, USA). Only good-quality sequences were selected for analysis. They were edited and aligned using BioEdit sequence alignment editor, version 7.2.6 (ref. 33), and ClustalW implemented in MEGA v6.06 (ref. 34). The downloaded 16S sequences of dung beetles from other regions available in NCBI and our sequences were used to construct the phylogeny tree. The final dataset included sequences of 411 base pairs.

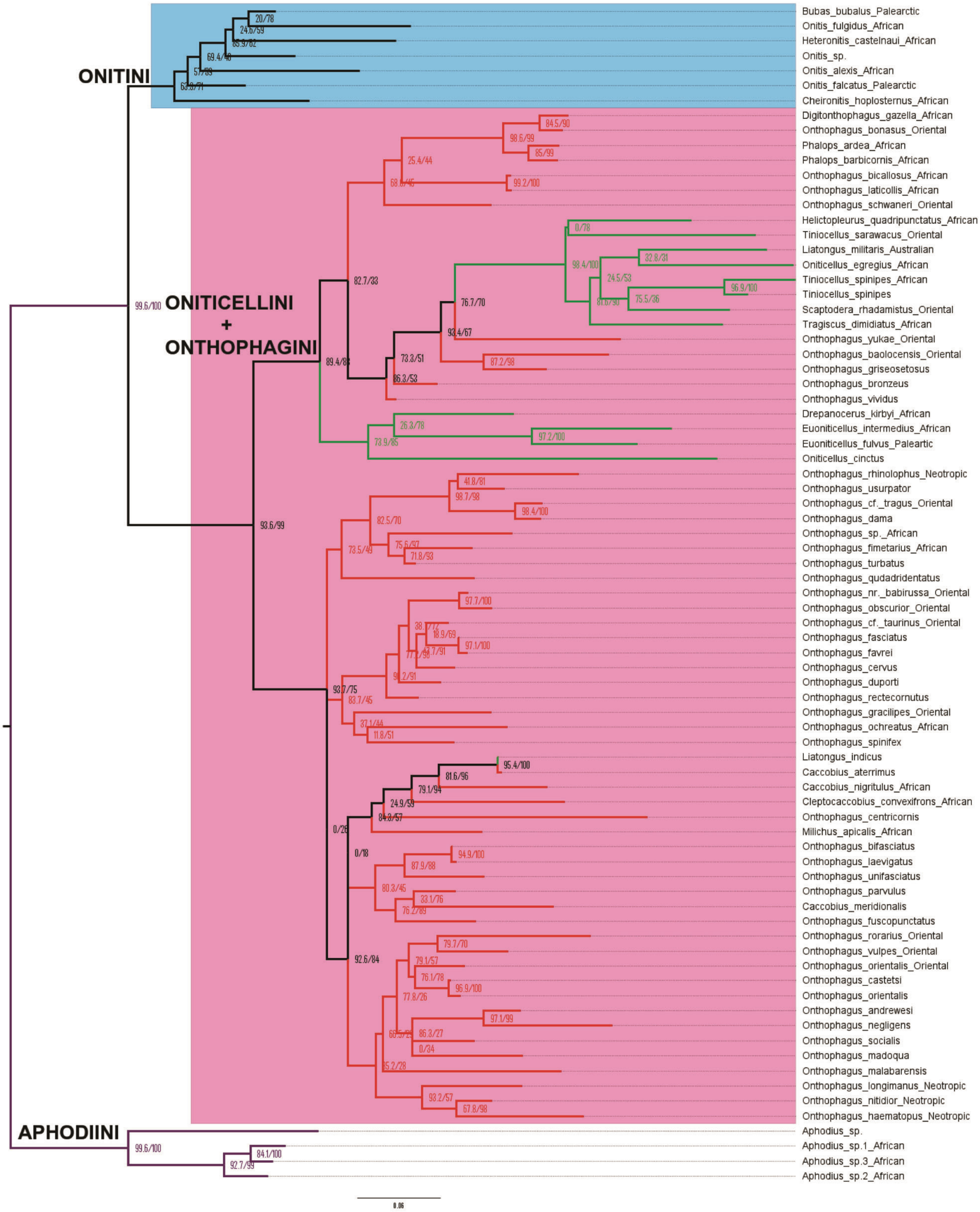

Figure 1. Maximum likelihood phylogeny inferred by IQTREE. Numbers beside nodes are IQTREE ultrafast bootstrap and SH-aLRT values. Species named without region name are sampled from the Western Ghats.

We selected the best-fit substitution model according to BIC using the model finder in IQTREE 1.6.12 (ref. 35). The tree searches were conducted with IQ-TREE 1.6.12 (ref. 36). Maximum likelihood trees with 1000 ultrafast bootstrap replicates and SH-aLRT test were performed using the GTR + F + I + G4 model. The phylogenetic trees were edited using Figtree v1.3.1 (ref. 37).

Sequences of 130 samples representing 26 *Onthophagus* species, 2 *Caccobius*, 1 *Tiniocellus*, 1 *Oniticellus*, 1 *Liatongus*, 1 *Onitis* and 1 *Aphodius* species were generated for 16S gene. Nine subgenera of *Onthophagus* were included in the analysis. We found that the tribe Oniticellini nested within the tribe Onthophagini in the phylogenetic trees (Figure 1). The non-*Onthophagus* genera within Onthophagini – *Caccobius*, *Cleptocaccobius*, *Milichus* – also nested within *Onthophagus*. However, *Digitonthophagus* and *Phalops* – the other two non-*Onthophagus* genera in Onthophagini, were distantly placed in a clade. Onitini, with all its genera included in the analysis: *Bubas*, *Cheironitis*, *Heteronitis* and *Onitis*, was distantly placed from Onthophagini + Oniticellini as a separate clade (Figure 1).

In this study, we inquired whether the tribal positions of Onthophagini, Onitini and Oniticellini on the phylogeny tree remain the same as observed by Breeschoten *et al.*[18], when the species of India – an under-represented, but important biogeographical region – was included the analysis. To the best of our knowledge, there have been no previous molecular phylogeny studies of dung beetles in India.

Balthasar[11,12] classified dung beetles into two distinct subfamilies: Coprinae and Scarabaeinae. The former subfamily included the tribes Coprini, Dichotomini, Phanaeini, Oniticellini, Onitini and Onthophagini, whereas the latter subfamily included the tribes Eucraniini, Eurysternini, Canthonini, Gymnopleurini, Scarabaeini and Sisyphini. Our study does not support this morphology-based tribal classification of Oniticellini and Onthophagini, as genera of Oniticellini nested within Onthophagini rather than grouping into two branches in the tree. The close relationship of Onitini to Onthophagini and Oniticellini has also been supported by several past studies[3,20,26–28]. In a study based on the morphological characters, Tarasov and Génier[10] showed that Onitini is distantly related to the other two tribes. We obtained a phylogenetic tree in which Onitini formed a separate clade, yet confirming the close relationship to Onthophagini and Oniticellini.

We were also interested to know the positions of all the genera of Onthophagini with respect to the genus *Onthophagus* in the phylogenetic tree. The nesting of *Caccobius* within *Onthophagus* questions the separate genus status for *Caccobius*[18]. Thus, our study supports that of Breeschoten *et al.*[18] and proposes a necessary change in the present classification of *Caccobius*. They also found that *Caccobius*, *Cleptocaccobius* and *Milichus* have nested within *Onthophagus*, and suggested that Onthophagini might not be monophyletic[18]. We also obtained similar results in the phylogenetic tree. As suggested by Breeschoten *et al.*[18], we recommend that all these factors may be considered while redefining the tribal status. Some researchers have revised Onthophagini based on morphological data and elevated the following subgenera of *Onthophagus*, *Digitonthophagus* Balthasar, 1959, *Progoderus* Lansberge, *Strandius* Balthasar, *Diastellopalpus* Lansberge, and *Euonthophagus* Balthasar into separate genera[24,38,39]. Together with such changes, the tribal-level classification should also be supported by a global analysis with sufficient sampling coverage and nodal support. Although our analysis is restricted to a maximum likelihood tree, tracing the evolutionary origin can give more insight into the evolution of dung beetles of the Western Ghats biodiversity hotspot. We caution that the results discussed here are indicative, since the study was based on a single mitochondrial marker gene. For conclusiveness, we recommend further explorative studies based on both nuclear and mitochondrial gene markers.

Overall, the present study agrees with the suggestions provided by previous molecular studies that the tribal-level classification needs revision. This study will enrich the molecular information on Indian dung beetles, which is currently lacking. It also calls for an in-depth phylogeny with all the species reported so far from India.

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