Phylogeny of the family Trogidae (Coleoptera: Scarabaeoidea) inferred from mitochondrial and nuclear ribosomal DNA sequence data

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Abstract. Trogidae constitute a monophyletic and biologically unique family within Scarabaeoidea, being the only keratinophagous group in the superfamily. Traditionally, the family has been divided into three distinctive genera, *Polynoncus* Burmeister, *Omorgus* Erichson and *Trox* Fabricius. Although the taxonomy of the group is relatively well studied, changes to the existing classification have recently been proposed and the family as currently constituted has not been subjected to phylogenetic analyses. Here we present a molecular phylogeny for this cosmopolitan family based on three partially sequenced gene regions: 16S rRNA, 18S rRNA and 28S rRNA (domain 2). Included in the analyses are representatives belonging to four of the five extant genera (and three of the four subgenera) from all major zoogeographic regions, representing about 20%of the known trogid species diversity in the family. Phylogenetic analyses performed included parsimony and Bayesian inference. We deduce their historical biogeography by using trogid fossils as calibration points for divergence estimates. Our analyses resolved relationships between and within genera and subgenera that are largely congruent with existing phylogeny hypotheses based on morphological data. We recovered four well-supported radiations: *Polynoncus*, *Omorgus*, Holarctic *Trox* and African *Phoberus* MacLeay. On the basis of this study, it is proposed that taxonomic changes to the generic classification of the family be made. The subgenera *Trox* and *Phoberus* should be elevated to genera to include the Holarctic and all the Afrotropical species, respectively, and *Afromorgus* returned to subgeneric rank. Estimates of divergence time are consistent with a Pangaean origin of the family in the Early Jurassic. The subsequent diversification of the major lineages is largely attributed to the break-up of Pangaea and Gondwana in the Middle Jurassic and early Late Cretaceous, respectively.

Introduction

Trogidae MacLeay (Coleoptera: Scarabaeoidea) comprise approximately 330 species worldwide (Scholtz, 1982; Smith, 2003; Pittino, 2006; Zidek, 2013). This family is well known for its remarkable feeding specialization; all known species (adults and larvae) feed almost exclusively on keratin and are the only members of the Scarabaeoidea capable of digesting it (Scholtz,

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1986a). They primarily inhabit the temperate and arid/savannah regions of the world. Africa and Eurasia have the richest fauna with about 100 species each, followed by Australia (55 species), and South and North America, with around 50 species each (Scholtz, 1982, 1986a,1986b, 1990; Pittino, 2006; Table 1). The group is taxonomically well studied and the fauna of each of the zoogeographical regions has been revised (Balthasar, 1936; Haaf, 1954a,1954b; Vaurie, 1955, 1962; Scholtz, 1980, 1982, 1986b, 1990; Pittino, 1983, 1985; Scholtz *et al.*, 2007; Zidek, 2013).

Much of our understanding of generic-level relationships within Trogidae is the result of Scholtz's (1986a) phylogenetic

Table 1. Classification and biogeographical distribution of the family Trogidae.

Genus	Subgenus	Region	Distribution	# spp.
Troginae				
Trox	*Trox*	Palaearctic	Europe/Asia/Oriental	77
Trox	*Trox*	Nearctic	North America	28
Trox	*Trox-Phoberus*	Afrotropical	Sub-Saharan Africa, Madagascar	40
Madagatrox	–	Afrotropical	Madagascar	1
Omorginae				
Omorgus	*Omorgus*	Australasian	Australia, Indonesia, Asia	57
Omorgus	*Omorgus*	Nearctic	North America	20
Omorgus	*Omorgus*	Neotropical	South America	14
Omorgus	*Haroldomorgus*	Neotropical	South America	1
Afromorgus	–	Afro-Oriental	Africa, Arabia, Orient, Asia	54
Polynoncus	–	Neotropical	South America	34

study, which was the first attempt to infer relationships among and within genera based on synapomorphic characters. The resulting phylogenetic classification, which has remained relatively stable for the last three decades, divided the family into two distinct lineages, a basal *Trox* Fabricius lineage (with two subgenera, *Trox* s.s. and *Phoberus* MacLeay) and a derived lineage consisting of the genera *Polynoncus* Burmeister and *Omorgus* Erichson (with tree subgenera *Omorgus* s.s., *Haroldomorgus* Scholtz and *Afromorgus* Scholtz). Phylogenetic studies that followed Scholtz (1986a) supported these findings, and demonstrated that: (i) Trogidae is a monophyletic group within the superfamily Scarabaeoidea; and (ii) monophyletic genera can be defined on the basis of both adult and larval synapomorphic character states (D'Hotmann & Scholtz, 1990; Nel

& Scholtz, 1990; Scholtz & Peck, 1990; Scholtz, 1991, 1993; Browne *et al.*, 1993; Browne & Scholtz, 1995, 1999; Grebennikov & Scholtz, 2004) (Fig. 1).

Subsequent authors have proposed changes to Scholtz's (1986a) classification. Nikolajev (2005) split Trogidae into two subfamilies: Troginae MacLeay containing the genus *Trox*, and Omorginae Nikolajev comprising the genera *Omorgus* and *Polynoncus*. Pittino (2006) elevated the subgenus *Afromorgus* to generic status. A new genus from Madagascar, *Madagatrox* Pittino, was recently described (Pittino, 2010). There are currently five valid extant genera in Trogidae: *Trox* (with two subgenera, *Trox* and *Phoberus*), *Omorgus* (with two subgenera, *Omorgus* and *Haroldomorgus*), *Afromorgus*, *Polynoncus*, and *Madagatrox*. However, the family as currently constituted have yet to receive any formal phylogenetic analyses.

Trogidae have long been considered to represent a plesiomorphic group within the Scarabaeoidea, whose origin extends back to the Upper Jurassic/Lower Cretaceous (Scholtz, 1986a; Scholtz & Chown, 1995). In Scholtz's (1986a) attempt to resolve the phylogenetic relationships within the family, based on shared derived morphological characters and current distribution patterns, he hypothesized that trogids evolved in central Pangaea, prior to the break-up of the supercontinent that formed Laurasia and Gondwana. Following the break-up of Pangaea, the lineage that eventually gave rise to *Trox* was restricted to Laurasia, and the ancestral *Omorgus* lineages to Gondwana. The ancestral *Omorgus* and *Polynoncus* lineages radiated on the Gondwana continents, while the *Trox* lineage radiated in the Holarctic and later another derived lineage (*Phoberus*) dispersed into Africa, along the temperate eastern highland mountain faunal exchange route. The recent discovery of Mesozoic fossils of Trogidae has confirmed the long-standing view that the family represents an ancient group of beetles within Scarabaeoidea (Browne & Scholtz, 1999; see Krell, 2007; Nikolajev, 2009 for fossil data). These fossil records also predate the only current (molecular) estimate for the divergence of the family, which is
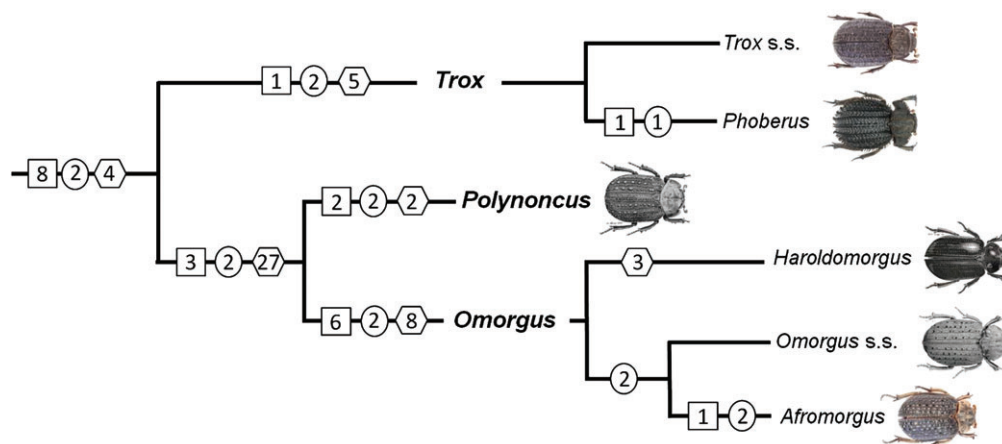

Fig. 1. Phylogram of the Trogidae genera and subgenera based on larval (boxes), adult (circles) and hind wing (hexagons) synapomorphic characters. Numbers represent the number of characters that unite each node. For details on larval characters, see Scholtz & Peck (1990), Scholtz (1993) and Grebennikov & Scholtz (2004); for adult (excluding hind wing) characters, see Scholtz (1986a) and Browne & Scholtz (1999); for hind wing characters, see Browne *et al.* (1993) and Browne & Scholtz (1995). Images depict the type species of the genus or subgenus.

121.4 million years ago (Ma; McKenna & Farrell, 2009). Furthermore, discovery of Cretaceous-aged fossils (98–144 Ma) ascribed to *Trox* from modern north-east Asia (Nikolajev, 2007, 2009) lends support to the proposed Laurasian origin for this lineage (Scholtz, 1986a).

The aim of this study is to investigate the phylogenetic relationships of the world genera and subgenera based on partial DNA sequences of three ribosomal gene regions (two nuclear and one mitochondrial). Secondly, we deduce their historical biogeography by estimating the ages of major divergence events using fossil data.

Materials and methods

Sampling, amplification, sequencing and processing of sequences

Specimens preserved in ethanol were obtained from all the major zoogeographic regions (Table S1), representing approximately 20% of the known trogid species diversity. Four of the five extant genera (and three of the four subgenera) in the family are represented in the ingroup sample. Only the rare, monotypic subgenus *Haroldomorgus* Scholtz and the monotypic genus *Madagatrox* are not included in our phylogeny, as specimens suitable for DNA extraction could not be obtained. Studies on the higher classification of Coleoptera and Scarabaeoidea indicate that Bolboceratidae Scholtz & Browne, Lucanidae Latreille and Trogidae are closely related clades within the superfamily Scarabaeoidea (Browne & Scholtz, 1999; Smith *et al.*, 2006; Hunt *et al.*, 2007; Lawrence *et al.*, 2011). Based on these results, representative species belonging to the families Bolboceratidae and Lucanidae were selected as suitable outgroups.

Collected individuals were identified, catalogued and stored for further data analysis. Specimens are deposited at the Department of Zoology and Entomology, University of Pretoria, South Africa (UPSA).

Total genomic DNA was extracted from all individuals using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, Penzberg, Germany). We amplified the nuclear genes 18S rDNA (18S) and 28S rDNA domain 2 (28S) and the mitochondrial gene 16S rDNA (16S) using previously reported primer sequences and a newly designed primer for 28S (Table S2). The 16S and 18S genes were amplified using the polymerase chain reactions (PCRs) performed in a final volume of 50μL. PCR reaction mixtures contained 2.5 mM $MgCl_2$, 20 pmol of each primer, 10 mM dNTPs, $1 \times$ PCR buffer, in the presence of one unit of TaqDNA polymerase (Super-Therm® DNA polymerase, Separation Scientific SA (PTY) LTD, South Africa) and 50–100 ng of genomic DNA template. For 28S, Emerald Amp®MAX HS PCRMastermix (Takara Bio Inc., Otsu, Shiga, Japan) was used. Different cycling parameters were implemented for each gene (Table S3). Successful amplifications were purified using the Roche High Pure Product Purification Kit (Roche Diagnostics) following the manufacturer's instructions.

To obtain DNA sequences, the cycle sequencing reactions were carried out in both directions using the BigDye®

Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Cycle sequencing products were precipitated using a standard sodium acetate/ethanol precipitation protocol. All sequences generated were viewed, assembled and edited in CLC BIO MAIN WORKBENCH version 5.5 (developed by CLC Bio, http://www.clcbio.com). New sequences (Table S1) were submitted to GenBank. Partial 16S RNA sequences of two more *Trox* species, *Trox uenoi* (Accession no.AB178316) and one unidentified species (accession no. EF487978) retrieved from GenBank, were included in the analysis.

Alignment

Processed sequences for each of the different gene regions (16S, 28S domain 2, 18S) were aligned based on secondary structure using RNASALSA version 0.8.1.win32 (Stocsits *et al.*, 2009). The program uses both secondary structure information for adjusting and refining sequence alignments and sequence information contained in the alignments to refine predictions of structure (Stocsits *et al.*, 2009). To initialize the analytical process, RNASALSA requires both an initial alignment of homologous RNA sequences and structural constraints (secondary structure predictions) for a single sequence to guide the alignment process.

The input alignments, for each gene region, were produced using MAFFT (Katoh & Toh, 2008). For 28S, structural alignment was performed based on the secondary structure of the 28S sequence of *Apis mellifera* (Gillespie *et al.*, 2006). The structural constraints for 18S (*Trox rudebecki* – accession number AY745581) and 16S (*T. uenoi* – accession number AB178316) were predicted using the RNAfold server (Hofacker, 2003; Gruber *et al.*, 2008). The server predicts minimum free energy (MFE) structures and base pair probabilities of single-stranded RNA or DNA sequences. Runs were initiated with default parameter settings for substitution cost, gap penalties and base-pairing occurrence stringencies, except for two stringency settings (-s1, -s3) which were set to 0.51. RNASALSA constructs a final multiple sequence alignment together with a consensus structure. For a detailed discussion of RNASALSA, refer to Stocsits *et al.* (2009). The alignments for the three partial sequences, including information on their consensus secondary structures, were concatenated into a supermatrix using the program FASCONCAT version 1.0 (Kück & Meusemann, 2010a,b).

Evaluating the general structure and potential conflict in alignments

We constructed a neighbour-network of the individual and the concatenated alignments using the program SPLITSTREE 4 (Huson & Bryant, 2006). Neighbour-net analyses can be used to visualize conflicting signal (ambiguities) in the data or alternative phylogenetic histories through a splits graph, and help to assess the tree-likeness (congruence) of the data, independently of tree reconstruction techniques (Huson & Bryant, 2006; Wägele & Mayer, 2007). Networks were based on

uncorrected *p*-distances. Parsimony uninformative sites were excluded. Bootstrap support values calculated were based on 1000 replicates.

Phylogenetic analysis

Analyses were conducted using maximum parsimony and Bayesian inference for the combined dataset. The parsimony analysis was implemented in PAUP*4.010b (Swofford, 2003) with the following heuristic search setting: all characters were equally weighted and unordered, gaps were treated as missing data and uninformative sites excluded; starting tree obtained via stepwise addition with random addition of sequences with ten replicates; branch-swapping = tree-bisection-reconnection; initial 'maxtrees' set to 200 with automatic increase by 100 and with 'MulTrees' option in effect. Where more than one most parsimonious tree was obtained, a strict consensus tree was calculated. Bootstrap support values (Felsenstein, 1985) calculated were based on 1000 replicates.

Bayesian analyses were performed in MRBAYES v3.1.2 (Ronquist & Huelsenbeck, 2003) and in PHASE v2.0 (Jow *et al.*, 2002; Gowri-Shankar & Jow, 2006). Both programs can simultaneously analyse partitioned datasets using both conventional DNA models and mixed RNA/DNA substitution models.

Analysis in MRBAYES was performed using the GTR + G model for 16S and 28S and the K80 + I + G model for 18S. The appropriate model of nucleotide evolution was estimated in JMODELTEST (Posada, 2008) under the Akaike information criterion (model parameters are summarized in Table S4). The data set was partitioned into three gene regions. Parameters for the different partitions were unlinked to obtain separate parameter estimates for each gene and the rate prior was set to variable. Flat Dirichlet priors were used in all analyses. Bayesian analyses were conducted by simultaneously running two Monte Carlo Markov (MCMC) chains for 15 million iterations. Trees were sampled every 200th iteration. The first 15 000 trees (20%) sampled were discarded as burn-in.

Analysis in PHASE was performed using the RNA7D + dG6 + I model (Tillier & Collins, 1998) for paired nucleotides. Although applied here empirically, as the testing of RNA models is beyond the scope of this study, the RNA7D model is a biologically plausible model for the evolution of paired nucleotide sites (Voigt *et al.*, 2008; Elven *et al.*, 2010; Gazave *et al.*, 2010). For the unpaired regions (loops), we used the general time reversible model (REV + dG6 + I). For further details of RNA-specific models, see Savill *et al.* (2001) and Jow *et al.* (2002). Phylogenetic trees were constructed using the *mcmcphase* module from the PHASE package with default values for priors and other *mcmc* parameters. The final analysis was run for 30 million iterations, and trees were sampled every 200 iterations. The first three million iterations were discarded as burn-in, which was a sufficient period for log-likelihood values and model parameters to reach stabilization. The module *mcmcsummarize* generated the consensus trees and all of the relevant statistics, including branch lengths and clade support for each node. Optimal branch lengths were calculated (as suggested in the PHASE manual) by

running an additional mcmcphase analysis (five million generations) under the same models as the 30 million generation *mcmcphase* run. We used the consensus tree from the final analyses (30 million generations) to fix the tree topology (with topology proposals turned off) and all other parameters left unchanged. To determine if all runs from the *mcmcphase* analyses reached the same stationary distribution, output files from the *mcmcphase* analyses were formatted, using a modified version of the Perl script phase2tracer.pl (original file available from http://hymenoptera.tamu.edu/rna/download.php) to create readable input files for TRACER 1.5 (Rambaut & Drummond, 2007). We repeated the analysis three times from random starting seeds, and compared the resulting topologies and posterior probabilities.

For comparative purposes, we tested a 16-state RNA model in MRBAYES, which implements a 16-state RNA stem substitution model (Schöniger & von Haeseler, 1994). This 16-state RNA model considers all possible base pairs as characters and assumes that compensatory base exchanges result from at least two substitution events (Voigt *et al.*, 2008). The concatenated dataset was partitioned into loop and stem regions for 16S, 18S and 28S. The covarian model was implemented for the paired nucleotides (stems). For the loop regions, a GTR + I + G (a six-state single-nucleotide model) was implemented. Parameters for the different partitions were unlinked to obtain separate parameter estimates for each gene and the rate prior was set to variable. Flat Dirichlet priors were used in all analyses. Posterior probabilities were calculated by simultaneously running two Markov chains for five million iterations. Trees were sampled every 200 iterations, and the first 5000 trees (20%) sampled were discarded as burn-in.

To evaluate the performance of the Markov chains, from MRBAYES analyses, we used the program AWTY (are we there yet?) (Nylander *et al.*, 2008) to assess the convergence rates of posterior probabilities and branch lengths from sampled trees. The program TRACER was used to monitor parameter stabilization [via inspection of estimated sample size (ESS) and graphical plots of parameter sampling]. FIGTREE v1.3.1 (Rambaut, 2009) was used to view all tree topologies. All Bayesian analyses were carried out on the Bioportal facility (http://www.bioportal.uio.no) at the University of Oslo, Norway.

Divergence analysis

We estimated the node ages for the major lineage-splitting events within Trogidae using the program BEAST v.1.6.2 (Drummond & Rambaut, 2007). We used a Bayesian relaxed molecular clock approach under the uncorrelated lognormal model, and the Yule speciation process was selected, for all data combined. The combined dataset was partitioned by gene. As models implemented in BEAST are not designed to take covariation into account, a separate GTR + G + I substitution was applied to each partition. Model and clock parameters were unlinked across partitions. The topology with optimized branch lengths, previously estimated using PHASE, was specified as a starting tree for the programme BEAST. Duplicate terminals were pruned from the

tree in TREEEDIT (Rambaut & Charleston, 2002) so that each species was only represented by a single individual.

Trogidae fossils from the Early Cretaceous serve as useful calibration points for divergence estimates (Krell, 2007; Nikolajev, 2009). We constrained the minimum age of the Omorginae (sensu Nikolajev, 2005) to the late Early Cretaceous (112 Ma) based on the fossil *Cretomorgus ikhbogdensis* Nikolajev (112.2–121 Ma). The potential minimum age for the Holarctic *Trox* was constrained to the Mid Cretaceous (98.9 Ma) based on the fossil *Trox minutus* Nikolajev (98.9–144 Ma). Calibrations were modelled under a lognormal distribution with an offset, mean and standard deviation, so that 95% of the prior distributions fall within the minimum and maximum ages of the fossils. Parameters of the lognormal distributions were as follows: for *T. minutus*, offset 98.9, mean 3.075, and standard deviation 0.42; for *C. ikhbogdensis*, offset 112.2, mean 1.52, and standard deviation 0.42. Two independent MCMC analyses were run for 60 million generations with parameters sampled every 2000 generation, discarding the first 6000 (20%) trees sampled from each run as burn-in. We kept default settings for all other priors and operators. The application LOGCOMBINER v1.6.2 (Drummond & Rambaut, 2007) was used to combine the log and tree output files from the two independent runs. TRACER was used to assess the convergence between runs. The module TREEAN-NOTATOR v1.6.2 (Drummond & Rambaut, 2007) generated the maximum clade credibility tree and calculated the mean ages (and 95% confidence intervals).

Results

The final combined molecular dataset consisted of 107 taxa and 1568 bp (base pairs): 16S = 405 bp; 18S = 562 bp and 28S (D2) = 601 bp. Of the 1568 base pairs, 730 (46.6%) were paired characters, 838 (53.4%) were unpaired and 421 characters were parsimony-informative (refer to Table S4 for data characteristics and model parameters). The heuristic search from the parsimony analysis produced 49 most parsimonious trees, with tree length = 1779, consistency index (CI) = 0.381 and retention index (RI) = 0.840. The strict consensus tree, with nodal support, is presented in Fig. 2. Neighbour-net analysis of the individual and the concatenated alignment are shown in Fig. 3. The consensus trees for the Bayesian analyses are shown in Fig. 4 and Figures S1 and S2. Summaries of the posterior probabilities (PPs) and bootstrap support (BS) from the neighbour-net, parsimony and Bayesian analyses, for nodes representing subfamilies, genera and subgenera recovered can be seen in Table 2.

Phylogenetic relationships

Four well-supported monophyletic lineages, *Polynoncus* (node D), *Omorgus* (node E), *Trox* (node H) and *Phoberus* (node I), were recovered across all methods of analyses (Figs 2, 4). However, tree topologies from the Bayesian analyses using mixed DNA/RNA nucleotide substitution models were overall better resolved (Fig. 4). The latter consistently resolved the

deeper phylogenetic relationships within the family, and recovered the two subfamilies, Omorginae (node B) and Troginae (node C) (sensu Nikolajev, 2005). The phylogenetic relationships hypothesized in the following were based on the results of the mixed DNA/RNA model analysis (Fig. 4, Figure S2), and were largely congruent with the existing morphological phylogeny (Fig. 1).

Phylogenetic relationships within Omorginae (node B) were well resolved (Fig. 4). Two monophyletic sister groups deemed equivalent to genera were recovered: *Polynoncus* (node D) and *Omorgus* (node E). The genus *Omorgus* comprised two poorly supported sister clades: *Omorgus* (node F), representing the New World and Australasian radiations; and *Afromorgus* (node G), comprising an Afro-Oriental radiation. The only Asian representative of *Afromorgus*, *Afromorgus pauliani* (Haaf), included in the study lay in the well-supported (node O) clade containing *Afromorgus squalidus* (Olivier), the type species of the genus.

Within *Omorgus* there were two distinct lineages: one representing a Neotropical + Nearctic group (node J) containing *Omorgus suberosus* (Fabricius), the type species of the genus and therefore also of the subgenus *Omorgus*; and a second, Neotropical + Australian group (node K). This latter clade included three ambiguously resolved clades that were biogeographically coherent but with sparse taxon sampling. One of these clades (node M) contains *Omorgus pastillarius* (Blanchard), the type species of a formerly recognized subgenus *Chesas* Burmeister. The Australian species *Omorgus gigas* Harold is the type species of the former subgenus *Megalotrox* Preudhomme De Borre and is a relative of *Omorgus tatei* (Blackburn), found in the well-supported (node N) sister clade containing *O. pastillarius* (Fig. 1). The third well-supported clade (node L) was also Australian.

Troginae (node C) is composed of two major monophyletic radiations; the Holarctic *Trox* (node H) and the Afrotropical *Phoberus* (node I). Nearctic, Palaearctic and Oriental *Trox* species were interspersed on the tree despite obvious geographical separation among species, and although taxon sampling was only 16 species of about 100 in the region, the posterior probabilities were generally reassuring. All of the Afrotropical species were grouped together, contradicting the independent morphological data (Scholtz, 1986a). Taxon sampling of the Afrotropical group was more than adequate; 24 out of 40 known species were included in the analyses and the monophyletic status of *Phoberus* is not expected to change with increased taxon sampling. The Madagascan species, *Trox perrieri* Fairmaire, currently placed in the subgenus *Trox*, was nested on a long branch within the Afrotropical *Phoberus* lineage.

Neighbour-net graph: general structure and conflict of alignments

The neighbour-network analysis for the individual and the combined dataset (Fig. 3) revealed the presence of four major clusters (*Polynoncus*, *Omorgus*, *Trox* and *Phoberus*). These were largely congruent with the topologies recovered from the parsimony and Bayesian analyses (Figs 3, 4). Combining the
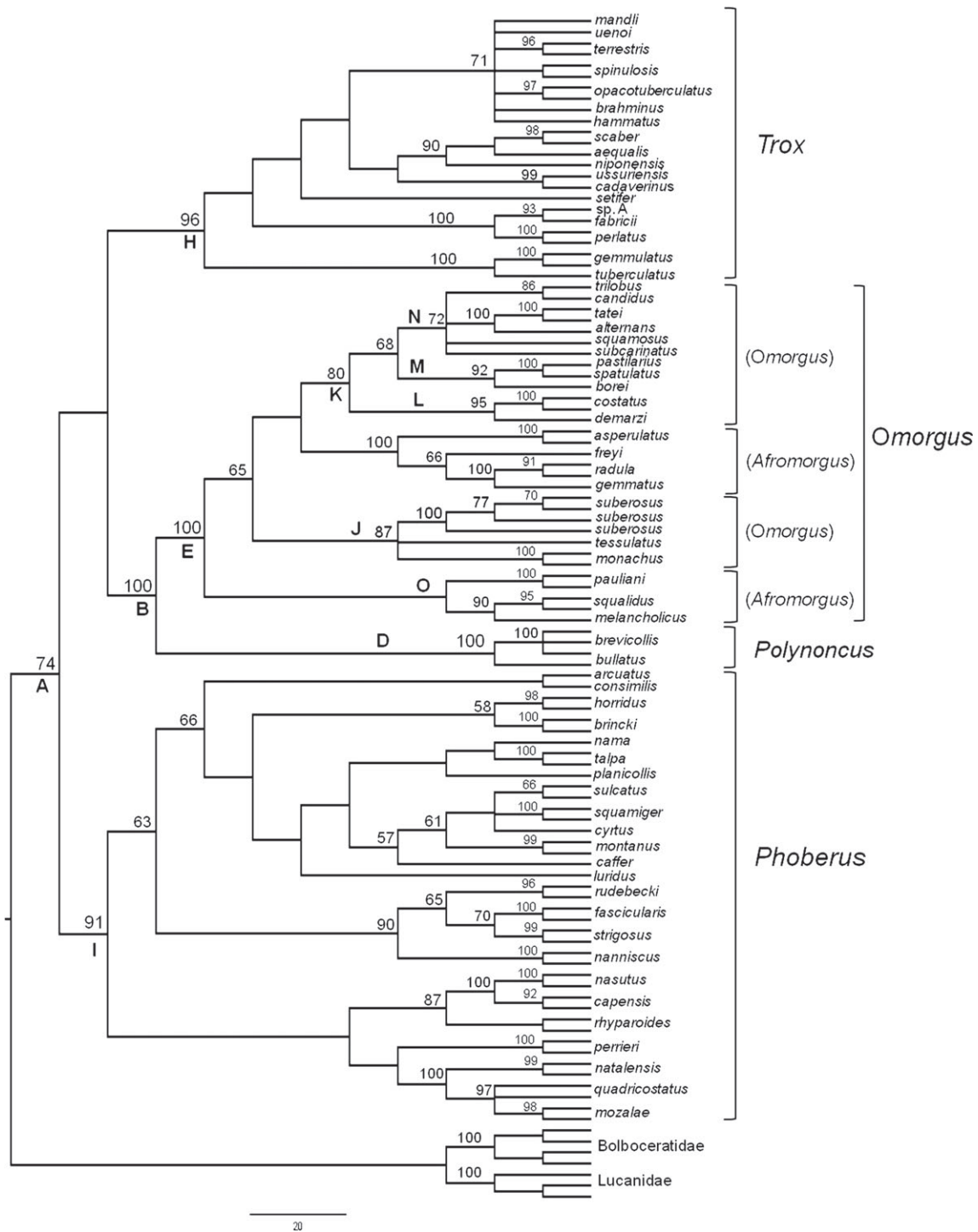
Fig. 2. Strict consensus phylogram for the Trogidae of 49 most parsimonious trees for the combined dataset with bootstrap support. Only bootstrap support ≥ 50 are shown.

individual datasets in a single analysis improved the split support for a large number of nodes in the splits graph. There was clear split support (long parallel edges) for *Polynoncus* being the sister group to *Omorgus*. The generic separation of *Afromorgus* and

Omorgus was not as well supported by the analysis, and *Afromorgus* showed evidence of encompassing disparate lineages. The networks showed conflicting splits between *Phoberus* and *Trox*, but the Madagascan endemic, *T. perrieri*, remained closely
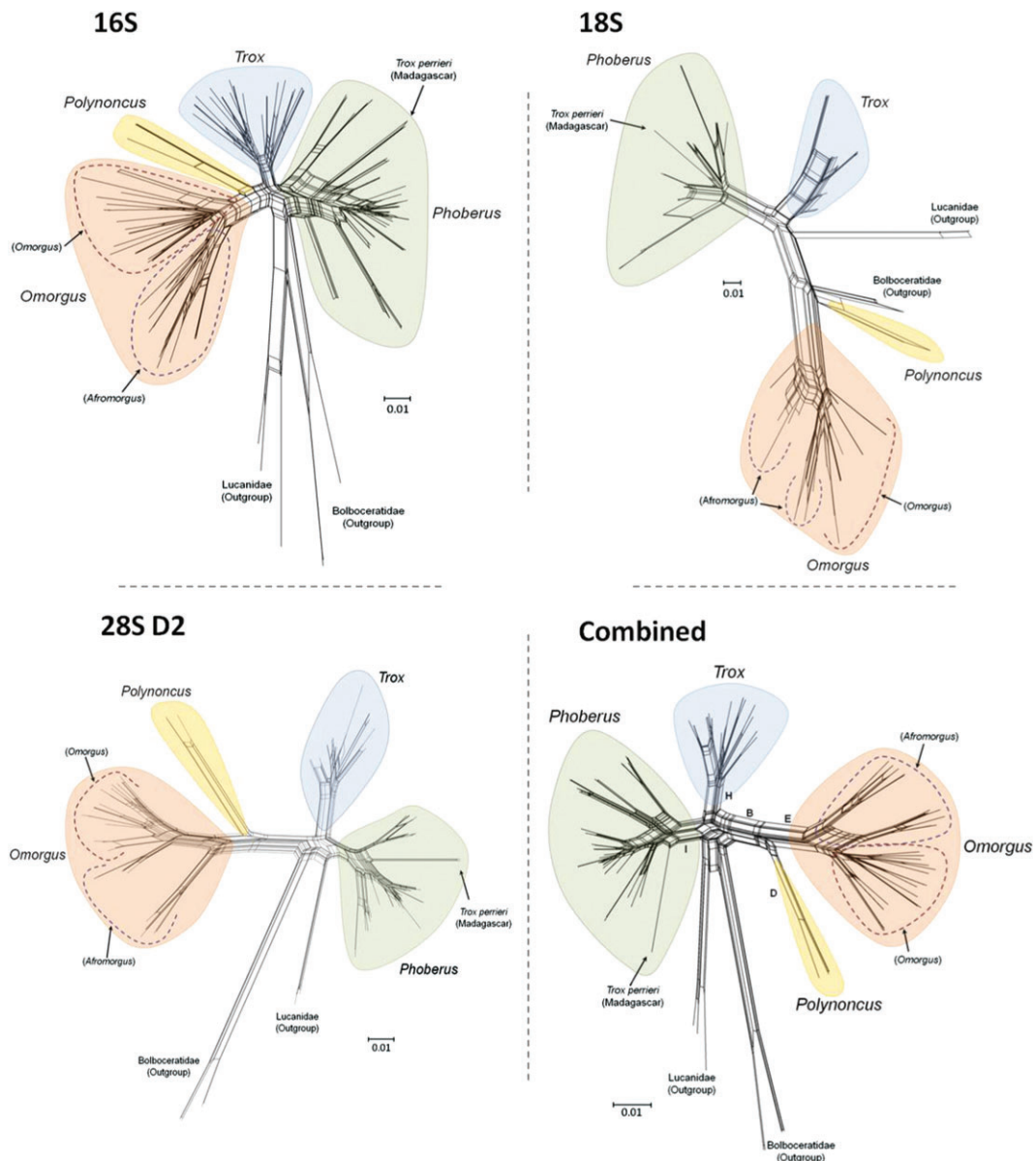
Fig. 3. Neighbour-net graph for the individual and combined molecular datasets, based on uncorrected *p*-distances.

related to the African *Phoberus*. The support for the major splits is summarized in Table 2.

Divergence time estimates

BEAST analysis (Fig. 5) dated the origin of Trogidae during the early Jurassic (194 Ma; 95% confidence interval: 152.7–250.1 Ma). The subfamilies Omorginae (node B) and Troginae (node C) diverged well before the Cretaceous (174 Ma; 139.7–210.4 Ma, well before the Cretaceous node A). The time of divergence between the Holarctic *Trox* (node H) and the Afrotropical *Phoberus* (node I) was estimated to have occurred

during the Late Jurassic (156 Ma; 124.54–189.1 Ma, node C). *Omorgus* and *Polynoncus* diverged from their last common ancestor at 117 Ma (113.63–122.78 Ma), also during the mid-Lower Cretaceous (node B). The estimated time of divergence between the subgenera *Omorgus* (node F) and *Afromorgus* (node G) was around 90 Ma (73.82–104.56 Ma, node E). The Australian and New World sister groups within *Omorgus* separated about 81 Ma (65.53–97.75 Ma, node F). The three ambiguously supported lineages subtending the Australian and Neotropical radiations occurred around the Cretaceous/Tertiary boundary (node K). The probable existence of ancestors of the Nearctic *Omorgus* (node J) at 46 Ma (25.97–67.79 Ma) predates the existing hypothesis of a Pliocene-Pleistocene colonization
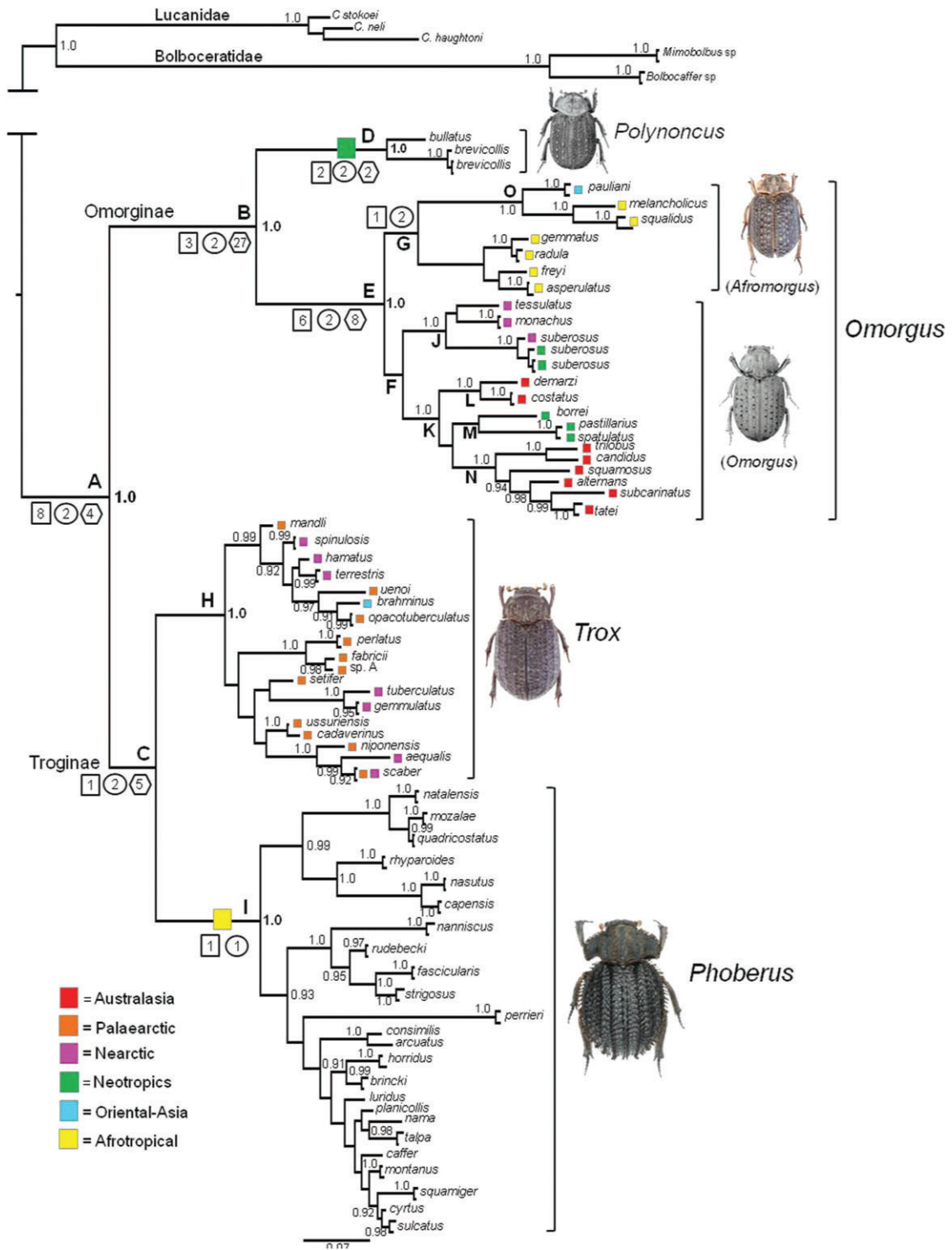
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Fig. 4. Bayesian topology from the PHASE analysis. Numbers next to each node are the posterior probability support. Only nodes with posterior probabilities ≥ 90% are shown. Images depict the type species of the genus or subgenus. Synapomorphic character sets (see Fig. 1) that unite each node are plotted on the phylogram.

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Table 2. Support values for nodes recovered representing subfamilies, genera and subgenera for the parsimony, Bayesian and splits graphs.

Node	Taxonomic group	BI (DNA) MRBAYES (PP)	BI (RNA/DNA model) MRBAYES (PP)	PHASE (PP)	MP PAUP (BS)	NN SPLITSTREE (BS)
A	Trogidae	0.69	1.00	1.00	74	–
B	Omorginae (sensu Nikolajev, 2005)	1.00	1.00	1.00	100	97
C	Troginae (sensu Nikolajev, 2005)	0.69	0.80	0.81	–	–
D	*Polynoncus*	1.00	1.00	1.00	100	100
E	*Omorgus*	1.00	1.00	1.00	100	99
F	(*Omorgus*)	0.83	0.71	0.71	–	–
G	*Afromorgus*	0.96	0.93	0.84	–	42
H	*Trox* s.s.	1.00	1.00	1.00	96	95
I	*Phoberus*	1.00	1.00	1.00	91	94
J	*Omorgus* (Neotropical + Nearctic group)	1.00	1.00	1.00	87	93
K	*Omorgus* (Neotropical + Australian group)	1.00	1.00	1.00	80	51
L	*Omorgus* (Australian group)	1.00	1.00	1.00	95	97
M	*Omorgus* (Neotropical group)	0.98	0.90	0.89	92	77
N	*Omorgus* (Australian group)	1.00	1.00	1.00	72	76
O	*Afromorgus* Asian representative	1.00	1.00	1.00	–	97

BI, Bayesian inference; MP, maximum parsimony; NN, neighbour-net; PP, posterior probability; BS, bootstrap support.

of North America (~ 2 Ma) from South America for this group (Scholtz, 1986a).

Discussion

Molecular phylogeny and the systematics of Trogidae

Our molecular phylogeny supports the morphological phylogeny proposed by Scholtz (1986a) and Scholtz & Peck (1990) in most aspects, and the subfamily divisions (Trogidae-Omorginae) of Trogidae proposed by Nikolajev (2005). Omorginae was the only subfamily recovered unambiguously. Although there was not particularly strong branch support for the monophyly of Troginae, there are convincing (larval and adult) morphological synapomorphic characters supporting the monophyly of Troginae and similarly for Omorginae (Scholtz, 1986a; Scholtz & Peck, 1990; Browne *et al.*, 1993; Nikolajev, 2005; Figs 1, 4).

Within Omorginae, the genera *Omorgus* and *Polynoncus* are recovered as monophyletic and the sister group relationship is clearly supported by both morphological and molecular data (Figs 1, 4). The genus *Omorgus* is divided into two groups: (i) *Omorgus*, representing the Australasian and New World species; and (ii) *Afromorgus*, containing all the Afrotropical and Asian species. *Afromorgus* is morphologically easily separated from *Omorgus* on the basis of two apomorphic characters on the male genitalia: (i) pars basalis fused dorsally; and (ii) median lobe complex, not simple (Scholtz, 1986a). Pittino (2006) considered the aforementioned characters important enough to elevate *Afromorgus* to generic status. However, our results clearly indicate that *Afromorgus* is a well-defined subgroup within the genus *Omorgus* and the generic separation of *Afromorgus* and *Omorgus* s.s. is not supported. We intuitively follow the classification system for the genus *Omorgus* and its subgenera, as proposed by Scholtz (1986a).

The subgenus *Omorgus* may well comprise several lineages equivalent to subgenera, but evidence for their monophyly is equivocal. Our results tentatively suggest resurrection of two former subgeneric names, *Megalotrox* and *Chesas*. The former is assignable to all Australian *Omorgus* species and the latter to some Neotropical representatives. Scholtz (1986a) was unable to provide any support for these species representing a monophyletic group in the morphological phylogenetic reconstruction of the family and synonymized it with *Omorgus*. Burmeister (1876) considered *O. pastillarius* unique among the Argentine species, on account of it being flightless, and placed it in the *Chesas* group which was later treated as a subgenus by Preudhomme de Borre (1886). *Chesas* was subsequently synonymized with *Omorgus* (Vaurie, 1962; Baker, 1968; Scholtz, 1986b). At present, we prefer not to recognize these groups formally without unequivocal evidence.

The subfamily Troginae consists of two monophyletic groups, the Holarctic *Trox* and the Afrotropical *Phoberus*. *Trox* are found in most of the major biomes of the Holarctic (Scholtz, 1982, 1986b; Pittino, 2006; Scholtz *et al.*, 2007). However, Palaearctic, Nearctic and Oriental groups show little phylogenetic separation, indicating probable repeated lineage-mixing across Beringia in various pulses, in response to glacial and interglacial episodes.

All the Afrotropical species group naturally together in *Phoberus*, contrary to morphological evidence that the subgenus may be polyphyletic (Scholtz, 1986a). The close relationship of the Madagascan *T. perrieri* to the African *Phoberus* points unequivocally to dispersal from Africa, across the Mozambique Channel, to Madagascar (Nazari *et al.*, 2011; Sole *et al.*, 2011; Samonds *et al.*, 2012). Haaf (1953) considered *T. perrieri* related to other members on the mainland, which is supported by molecular evidence.

MacLeay (1819), in addition to proposing the family name, described the genus *Phoberus* to accommodate the large flightless southern African species, *Trox horridus* Fabricius. However,
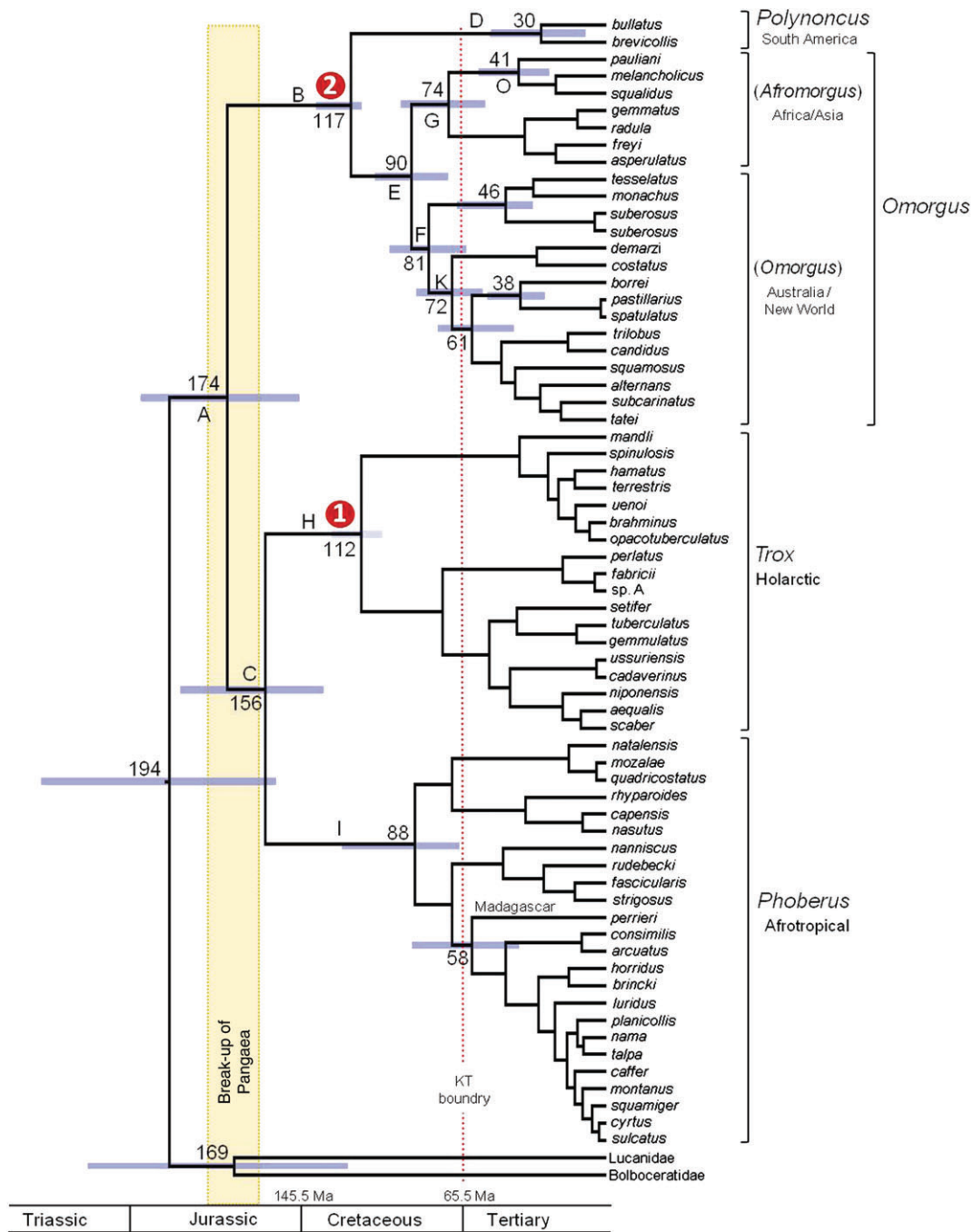
Fig. 5. Maximum clade credibility chronogram for Trogidae from the BEAST analysis. Age estimates for the major divergence events (in millions of years) with 95% highest posterior density intervals are depicted by node bars. Node labels 1 and 2 indicate fossil constraints.

Phoberus was not generally recognized as a genus; Burmeister (1876) and Preudhomme De Borre (1886) considered *Phoberus* to represent a subgenus, including only the type species. Harold (1872) and Scholtz (1979) considered *Phoberus* a synonym of the genus *Trox*, whereas Haaf (1953) treated *Phoberus* only as a species group. Péringuey (1900), Arrow (1912) and, later, Scholtz (1980, 1982) treated *Phoberus* as a subgenus of *Trox*, but

they differed in their views regarding inclusion of taxa in the subgenus. The results of the molecular phylogeny justify reinstating the genus *Phoberus* MacLeay to accommodate all Afrotropical (sub-Saharan) species.

The contemporary classification of this family has been based exclusively on morphological characters. Our molecular phylogeny, the first for the family, provides strong support

for the relationships between morphologically described genera and subgenera. On the basis of this study, it is now proposed that certain taxonomic changes to the generic classification of the family be made. The phylogenetic classification proposed closely follows the classification of Scholtz (1986a), except that we support Nikolajev's division of Trogidae into two subfamilies, Omorginae and Troginae, and suggest that the subgenera *Trox* and *Phoberus* be elevated to genera, with *Afromorgus* being returned to subgeneric rank. The Madagascan species, *T.* (*Trox*) *perrieri*, is transferred to *Phoberus*. The taxonomic changes we propose here will be formalized in a separate publication.

Historical biogeography

The divergence dating analysis proposed a Jurassic origin for extant Trogidae. Although it is difficult to identify the exact area of origin of Trogidae, we consider modern north-east Asia as an important candidate centre for their evolution. Mesozoic fossils assignable to Troginae and Omorginae (Krell, 2007; Nikolajev, 2009) and various basal Scarabaeoidea (Nikolajev, 2008a-d, 2010; Bai *et al.*, 2010, 2012, 2013; Nikolajev & Ren, 2010) support this view.

The north–south split of Pangaea (180–160 Ma) is considered a key geological event in the diversification of the extant Trogidae (Scholtz, 1986a). The divergence between the two subfamilies Omorginae and Troginae coincides with the initial fragmentation of the supercontinent during the Middle Jurassic, whereas the divergence of Holarctic *Trox* and African *Phoberus* corresponds to the final opening of the Tethyan seaway that eventually separated the two landmasses in the Late Jurassic (Stanley, 1986).

The evolution of *Omorgus* and *Polynoncus* proposed by Scholtz (1986a) remains plausible according to our results. Ancestral Omorginae radiated extensively on West Gondwana following the fragmentation of the supercontinent. Scholtz (1986a) hypothesized that the ancestor of *Omorgus* + *Afromorgus* diverged vicariantly when Africa and South America separated. This implies that the two lineages would have diverged in the Late Cretaceous. Our estimate for divergence between the two subgenera (~ 90 Ma), although it post-dated the presumed vicariant event (at about 100 Ma), is compatible with early Late Cretaceous separation of Africa from South America (Sereno *et al.*, 2004; Van Bocxlaer *et al.*, 2006). The alternative scenario is that speciation occurred more recently following long-distance dispersal from South America to Africa. The credibility interval (73.82–104.56 Ma) is wide enough to allow for both scenarios. Although long-distance dispersal is possible (e.g. Price *et al.*, 2011), the morphological differences between *Afromorgus* and *Omorgus* are substantial, reflecting a long period of isolation between the two groups. We tentatively conclude that *Afromorgus* and *Omorgus* diverged vicariantly when Africa and South America finally separated during the Late Cretaceous. The radiation of *Afromorgus*, estimated at 74 (90–54) Ma, occurred after the separation of Africa from South America, considering that the subgenus is absent

from the New World and Australasian Region. *Afromorgus* occurs throughout the Afrotropics and extends into Arabia, the Oriental and Indo-Malayan regions (Scholtz, 1980, 1986a).

The biogeography of the New World and Australasian *Omorgus* is complex and appears to be the result of post-Gondwana dispersal. The dispersal of ancestral *Omorgus* from South America into North America probably occurred during the Late Cretaceous to the Early Eocene when the two continents were connected across the proto-Caribbean archipelago, which formed when South America rifted from Africa (Sanmartín & Ronquist, 2004). There is evidence that the archipelago served as an important biotic exchange route (i.e. the first invasion of marsupial and placental mammals) since the mid-Cretaceous (Feller & Hedges, 1998; Sanmartín & Ronquist, 2004; Ortiz-Jaureguizar & Pascual, 2011).

A post-Gondwanan Antarctic connection between southern landmasses probably facilitated dispersal of South American *Omorgus* into Australia (Trewick, 2000; Almeida *et al.*, 2012). Divergence estimates are congruent with the time period when Australia, Antarctica and South America remained connected. The trans-Antarctic corridor served as an important dispersal route for the exchange of southern temperate biota between South America and Australia, since the Late Cretaceous and until the Early Palaeogene (Lawver *et al.*, 1992; Woodburne & Case, 1996; Sanmartín & Ronquist, 2004; Almeida *et al.*, 2012; see also Ali & Aitchison, 2009). Biotic exchange between southern landmasses was eventually disrupted by global cooling and the physical separation of the continents (Upchurch, 2008; Almeida *et al.*, 2012).

Polynoncus forms a well-defined group that evolved from an *Omorgus*-like ancestor in South America. The group is endemic to the continent, and radiation into the temperate regions probably occurred only after continental fragmentation. The formation of the Andes mountain range probably provided the temperate biome preferred by members of this genus, the majority of which are restricted to the southern one-third of the continent (Patagonian sub-region) (Scholtz, 1990).

Our results suggest a different evolution for the *Trox* and *Phoberus* from that proposed by Scholtz (1986a). Troginae may have diverged vicariantly when the opening of the Tethyan seaway finally separated Laurasia and Gondwana. The lineage confined to Africa gave rise to *Phoberus*, and the Laurasian lineage to *Trox*. According to the fossil evidence, modern East Asia was most likely the ancestral area of origin for extant *Trox*, which lends support to the proposed Laurasian origin for this genus (Scholtz, 1986a; Krell, 2007; Nikolajev, 2008a, 2009). *Phoberus* is endemic to continental Africa, which is probably a direct result of the geographic isolation of the African continent from the Middle Cretaceous to the Early Miocene following the fragmentation of Gondwana (Gheerbrant & Rage, 2006). The presence of *Phoberus* in Madagascar is best explained by sweepstakes dispersal (Rabinowitz & Woods, 2006; Ali & Huber, 2010). Out-of-Africa dispersal to the island during the Palaeogene (60 to 20 Ma) period was largely facilitated by ocean currents that flowed from the mainland towards Madagascar (Rabinowitz & Woods, 2006; Ali & Huber, 2010, Samonds *et al.*, 2012). Trans-oceanic dispersal from Africa to Madagascar

has been successfully invoked for various groups of mammals, amphibians, reptiles, insects (including dung beetles) and plants (Yoder & Nowak, 2006; Sole *et al.*, 2011; Townsend *et al.*, 2011; Zhou *et al.*, 2012).

Conclusion

In this study, we have provided the first molecular phylogeny for this group and successfully resolved relationships between and within genera and subgenera for the family. One of the more surprising results was the monophyly of *Phoberus*. Based on the evidence, we propose reinstating the genus *Phoberus* to accommodate all Afrotropical (including Madagascan endemic) species. The results re-emphasize the validity of the classification system for the genus *Omorgus* and its subgenera, as proposed by Scholtz (1986a). The study provides evidence of the Madagascan trogid fauna having an African origin. Colonization of the island probably occurred via dispersal from the mainland as opposed to ancient vicariant events. Divergence analysis was able to date the major events for the origin of extant lineages of Trogidae. The subsequent diversification of the major lineages is largely attributed to Pangaea and Gondwana vicariance events in the Mid-Jurassic and early Late Cretaceous, respectively. The separation of the landmasses resulted in the evolution of four distinct groups: *Omorgus*, *Polynoncus*, *Trox* and *Phoberus*. Phylogenetic sub-division within the family indicates a complex evolutionary history for the four major groups, and further studies should investigate the regional biogeography of the major lineages. Trogidae constitute a monophyletic and biologically unique family within Scarabaeoidea. It is clear that the switch to keratin feeding very early in scarabaeoid evolution provided trogids with a readily available food source that is mostly free of competitors, and this has remained the case since their initial radiation. To this day they are among the very last of the succession of insects that colonize and feed on animal remains, an adaptation that has endured and stood them in good stead since the Jurassic.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12074

Figure S1. Phylogeny of Trogidae inferred from the combined 16S, 28S domain 2 and 18S (sequence-based aligned) dataset and implementing a conventional DNA model setup using MrBayes.

Figure S2. Phylogeny of Trogidae inferred from the combined 16S, 28S domain 2 and 18S (sequence-based aligned) dataset and implementing a mixed RNA/DNA (16-state RNA) model setup using MRBAYES.

Table S1. List of taxa used in this study along with their GenBank accession numbers and locality data. X, indicates that PCR amplification failed.

Table S2. Summary of oligonucleotide primers used in this study.

Table S3. PCR thermal cycling profiles.

Table S4. Data characteristics and estimated model parameters for 16S, 28S domain 2, 18S and combined dataset.

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Supporting information

Figure S1. Phylogeny of Trogidae inferred from the combined 16S, 28S domain 2 and 18S (sequence-based aligned) dataset and implementing a conventional DNA model setup using MrBayes.
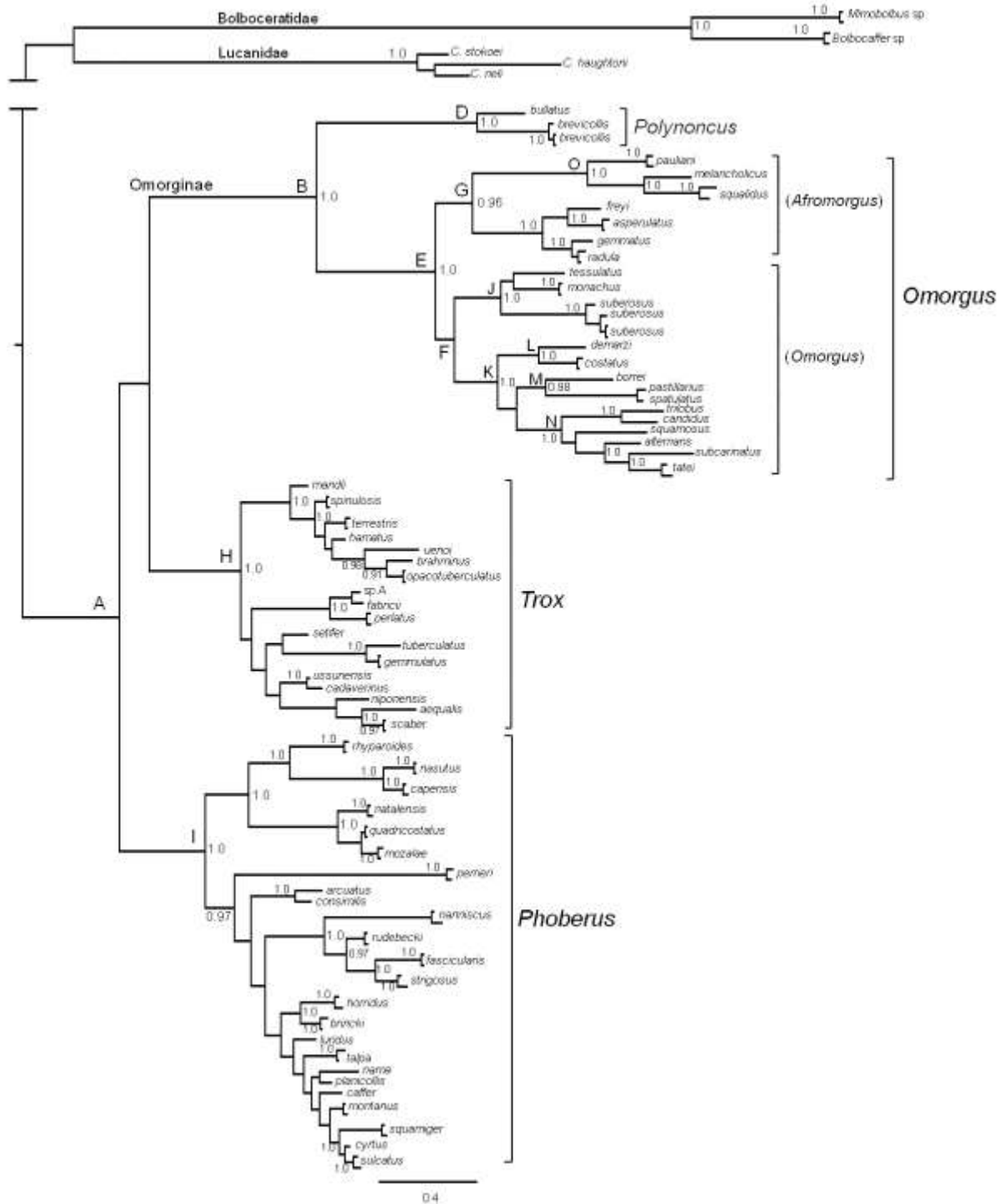
Figure S2. Phylogeny of Trogidae inferred from the combined 16S, 28S domain 2 and 18S (sequence-based aligned) dataset and implementing a mixed RNA/DNA (16-state RNA) model setup using MrBayes.
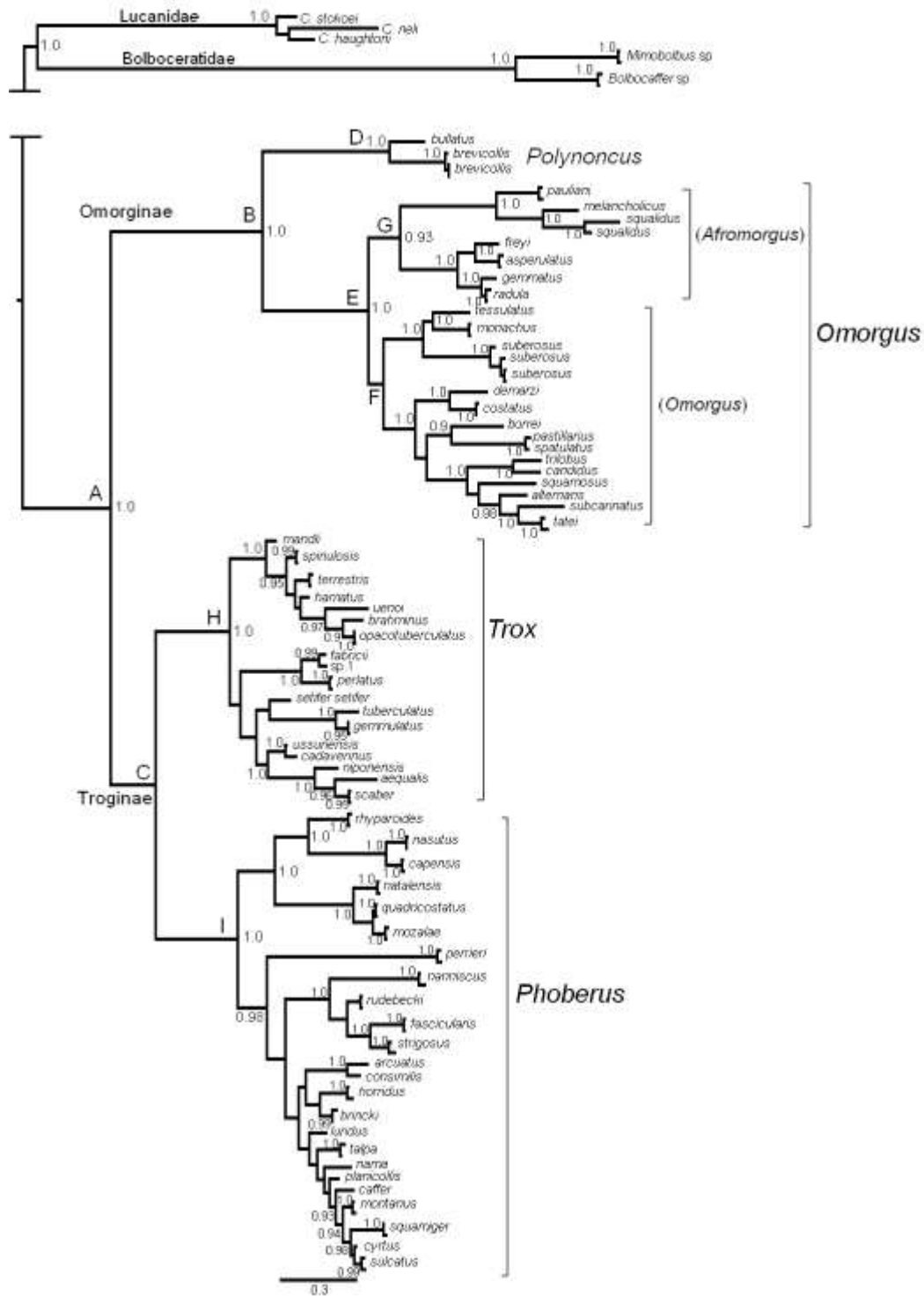
Table S1. List of taxa used in this study along with their GenBank accession numbers and locality data. X - Indicates PCR amplification failed.

Genus				GenBank Accession Numbers		
Species	Locality	ID	16S	28S (D2)	18S
Bolboceratidae (Outgroup)						
Mimobolbus sp.	Botswana: Kgalagadi Transfrontier Park, January 2005, C. Deschodt & P. Tshikae	B1	EF57 0408	KC80 1328	KC80 1189
Mimobolbus sp.	Botswana: Kgalagadi Transfrontier Park, January 2005, C. Deschodt & P. Tshikae	B2	EF57 0409	KC80 1329	KC80 1188
Bolbocaffer sp.	Botswana: Kgalagadi Transfrontier Park, January 2005, C. Deschodt & P. Tshikae	B3	EF57 0410	KC80 1330	KC80 1187
Bolbocaffer sp.	Botswana: Kgalagadi Transfrontier Park, January 2005, C. Deschodt & P. Tshikae	B4	EF57 0406	KC80 1331	KC80 1186
Lucanidae (Outgroup)						
Colophon neli	South Africa: Western Cape Prov., Swartberg Mt. Range, January 2008, W.P. Strümpher & C. Deschodt	C1	KC76 3267	KC80 1332	KC80 1232
C. haughtoni	South Africa: Western Cape Prov., Hexriver Mountains, January, 2008, W.P. Strümpher & C. Deschodt	C2	KC76 3256	KC81 0333	KC80 1233
C. stokoei	South Africa: Western Cape Prov., Hottentots-Holand Mt. Range, December 2007,W.P. Strümpher & C. Deschodt	C3	KC76 3248	KC80 1334	KC80 1234
Polynoncus						
P. brevicollis	Chile: San Antonio; Aguas Buenas; 3.IX.1999; Leg. V. Manuel Dieguez M.	3A	EF57 0366	KC80 1325	KC80 1221

P. brevicollis	Chile: San Antonio; Aguas Buenas; 3.IX.1999; Leg. V. Manuel Dieguez M.	3B	EF57 0373	KC80 1326	KC80 1220
P. brevicollis	Chile: San Antonio; Aguas Buenas; 3.IX.1999; Leg. V. Manuel Dieguez M.	3C	EF57 0385	X	X
P. bullatus	Chile: Curico; Rauco-fundo La Pancora; 31.VII.1999; Leg. V. Manuel Dieguez M.	1	EF57 0378	KC80 1327	KC80 1222

Omorgus
(*Omorgus*)

O. suberosus	Argentina: La Roja; Aimogasta; Ruto 60; S28°35' W66°44'; dead horse; 25.II.2002; Medina & Scholtz	5A	EF57 0389	KC80 1309	KC80 1197
O. suberosus	Argentina: La Roja; Aimogasta; Ruto 60; S28°35' W66°44'; dead horse; 25.II.2002; Medina & Scholtz	5B	EF57 0386	KC80 1308	KC80 1195
O. suberosus	Argentina: La Roja; Aimogasta; Ruto 60; S28°35' W66°44'; dead horse; 25.II.2002; Medina & Scholtz	5C	EF57 0393	X	KC80 1194
O. suberosus	USA: Arizona, Santa Cruz County; Pena Blanca Canyon; 17.VIII.2002; W. Moore	8	EF57 0370	KC80 1310	KC80 1196
O. spatulatus	Argentina: Salinas de Nihuil, 7.III.2002	4	KC80 1132	KC80 1305	KC80 1204
O. borrei	Argentina: Formosa, Laguna Yema. S24°19'54" W61°17'73", 11.XII.2008, F.C. Ocampo, G. SanBlas, F. Campon.	FE2	KC80 1118	KC80 1302	KC80 1202
O. pastillarius	Argentina: Mendoza, Molargile; 1049m; S35°53'21" W68°37'34"; F. Escobar	FE1	KC80 1133	KC80 1306	KC80 1205
O. tesselatus	USA: Coll. S. Whipple, 2009	Otes 01	KC80 1117	KC80 1313	KC80 1193
O. monachus	USA: Alabama, Madison County, Huntsville, Monte Sano State Park, 19.V.2005, Paul K. Lago	91	KC80 1116	KC80 1311	KC80 1198
O. monachus	USA: Mississippi, Coahoma County, Mississippi River; 10mi WNW, Clarkesdale, 10.V.2005, J. King	93C	KC80 1115	KC80 1312	KC80 1199
O .candidus	Australia: Yarramulla HS.; Undara Nat. Park, Qld.; 9.ii.2003; G. Monteith	12B	EF57 0379	KC80 1296	KC80 1206

O. costatus	Australia: Qld: 26°23'Sx146°12'E; Charleville, 5km NW; 3-5. Mar.2003, 310m, G. Monteith, C. Burwell, Mulga 51123	19A	EF57 0390	KC80 1304	KC80 1219
O. costatus	Australia: N Qld, Gilbert River nr. Georgetown; 4.ii.2003; J. Hasenpusch	10	EF57 0391	KC80 1303	KC80 1218
O. trilobus	Australia: Qld, Charleville, Ward River Crossing, 4.III.2003, G. Monteith	16	KC80 1119	KC80 1295	KC80 1207
O. demarzi	Australia: Qld: 26°23'Sx146°12'E; Charleville, 5km NW; 3-5 Mar 2003, 310m; G. Monteith, C. Burwell; Mulga 51123	17	EF57 0380	KC80 1307	KC80 1203
O. squamosus	Australia: NEQ: 17°16'S 145°52'E, Bellenden Ker Tap Stn. 17-18.Apr.1997. Monteith, Russel & Ovendan, 1560m	OA1	KC80 1123	KC80 1301	KC80 1211
O. alternans	Australia: 31°33'33"S, 115°38'21"E, Yanchep, West. Aus., 6.XI.2010, M. Keady	OA2	KC80 1122	KC80 1297	KC80 1217
O. subcarinatus	Australia: Carnarvon, W.A., 21.III.2011, P. Hutchinson, A. Sundholm	OA3	KC80 1124	KC80 1298	KC80 1201
O. tatei	Australia: Wadana Nature Reserve, ca 90km NE Geraldon, WA, S28°07' 30" E115°11'11", 16-17.IX.2009, T.F. Houston	4W	KC80 1120	KC80 1299	KC80 1200
O. tatei	Australia: Wadana Nature Reserve, ca 90km NE Geraldon, WA, S28°07' 30" E115°11'11", 16-17.IX.2009, T.F. Houston	5W	KC80 1121	KC80 1300	X

Afromorgus

A. pauliani	Laos: Vientiane Nat. University Campus, 18°2,534''N 102°37.768''E, 2-5.VI.2008, leg. S. Tarasov	1W	KC80 1127	KC80 1323	KC80 1214
A. pauliani	Laos: Vientiane Nat. University Campus, 18°2,534''N 102°37.768''E, 2-5.VI.2008, leg. S. Tarasov	2W	KC80 1128	KC80 1324	KC80 1213
A. gemmatus	Senegal: Mbour, VIII.1995	Ogem01	KC80 1131	KC80 1319	KC80 1209

A. squalidus	South Africa: Kgalagadi Transfrontier Park; S26°24'29.4" E20°42'32.7", 913m; P. Tshikae	73A	EF57 0407	KC80 1320	KC80 1215
A. squalidus	South Africa: Freestate Prov., Ladybrand, 1700m, 24.X.2007, S29.14971° E27.40688°, WP Strümpher	Oms q01	KC80 1125	KC80 1321	KC80 1216
A. asperulatus	Botswana: Nossob Camp; Kalahari Gemsbok Park; 25.I.2003	31	EF57 0412	KC80 1315	KC80 1190
A. asperulatus	Botswana: Kgalagadi Transfrontier Park; S26°24' 29.4" E20°42'32.7"; 913m; P. Tshikae	102A	EF57 0411	KC80 1314	KC80 1191
A. radula	South Africa: Kruger National Park, Skukuza Camp, 12.II.2003	38A	EF57 0387	KC80 1318	KC80 1210
A. radula	Botswana: North Central Kalahari, P. Tshikae	105	KC80 1130	KC80 1317	KC80 1208
A. melancholicus	South Africa: KwaZulu-Natal, Ndumu Game Reserve, XI. 2002, J. Harrison	49A	KC80 1126	KC80 1322	KC80 1212
A. freyi	Botswana: Kalahari Gemsbok Park, Nossob Camp, 25.i.2003	27	KC80 1129	KC80 1316	KC80 1192

Trox (*Trox*)

T. terrestris	USA: Florida, Archbold, Biol. Stat., nr L. Placid; 22-23.xi.2002; V. Grebennikov	7A	EF57 0383	KC80 1283	KC80 1171
T. terrestris	USA: Florida, Archbold, Biol. Stat., nr L. Placid; 22-23.xi.2002; V. Grebennikov	7B	EF57 0394	KC80 1282	KC80 1168
T. gemmulatus	Mexico: Chihauhau; Creel, 11.VII.2002; W. Moore	9A	EF57 0363	KC80 1290	KC80 1175
T. gemmulatus	Mexico: Chihauhau; Creel, 11.VII.2002; W. Moore	9B	EF57 0359	KC80 1291	KC80 1174
T. aequalis	USA: : Alabama; Madison County; Huntsville; Monte Sano State Park; 21.V.2005; Paul K. Lago	90	EF57 0375	X	KC80 1181
T. hamatus	USA: New Jersey; Somerset Co.; Hutcheson Memorial Forest; 3-5.VIII.2004; pitfall w/ dog faeces	83B	EF57 0381	KB80 1278	KC80 1169
T. tuberculatus	USA: Mississippi; Coahoma County; Mississippi River; 10mi WNW Clarksdale; 10.V.2005; Jonas King	95	EF57 0364	KC80 1292	KC80 1173

T. spinulosis	USA: Mississippi; Coahoma County; Mississippi River; 10mi WNW Clarksdale; 10.V.2005; Jonas King	96A	EF57 0362	KC80 1279	KC80 1170
T. spinulosis	USA: Mississippi; Coahoma County; Mississippi River; 10mi WNW Clarksdale; 10.V.2005; Jonas King	96B	EF57 0376	KC80 1280	KC80 1172
T. fabricii	Greece: Naroc - Nassa, 15.III.2011, O. Boilly	Tfab	KC80 1110	KC80 1293	KC80 1184
T. scaber	UK: Great Britain, Oxford	TSc01	KC80 1114	KC80 1288	KC80 1179
T. scaber	UK: Great Britain, Oxford	TSc02	KC80 1113	KC80 1287	KC80 1182
T. perlatus	Spain: Andalusia Camping Torre del Oro (ca 25km SE Huelva); 37°05'41.9"N 06°43'44.1"W; 18.V.2006; D. Ahrens & S. Fabrizi	TpE1	KC80 1111	KC80 1294	KC80 1185
T. perlatus	Spain: Andalusia Camping Torre del Oro (ca 25km SE Huelva); 37°05'41.9"N 06°43'44.1"W;18.V.2006; D. Ahrens & S. Fabrizi	TpE2	KC80 1112	X	KC80 1183
T. brahminus	Laos: Bolaven Plateau, Bam. Thongray, 15°14.054'N 106°31.867'E, 1000m, 8-16.VI.2008, S. Tarasov	TbA1	KC80 1106	KC80 1275	KC80 1167
T. cadaverinus	China: Mt.Zheng-nan-gou, Wuchang City, Heilongjiang, 1-11.VI.2011, Li Jingke	Tcad 01	KC80 1108	KC80 1285	KC80 1178
T. mandli	China: Mt.Zheng-nan-gou, Wuchang City, Heilongjiang, 1-11.VI.2011, Li Jingke	Tdo	KC80 1107	KC80 1281	X
T. niponensis	Japan: Nara-ken; Kamikitayama-mura; Mt. Wasamatayama; 5.VI.2005; Satoru Nu leg.	100	EF57 0413	KC80 1289	KC80 1180
T. opacotuberculatus	Japan: Nara-ken; Kamikitayama-mura; Mt. Wasamatayama; 5.VI.2005; Satoru Nu leg.	101A	EF57 0382	KC80 1277	KC80 1165
T. opacotuberculatus	Japan: Nara-ken; Kamikitayama-mura; Mt. Wasamatayama; 5.VI.2005; Satoru Nu leg.	101B	EF57 0368	KC80 1276	KC80 1166

T. ussuriensis	China: Mt.Zheng-nan-gou, Wuchang City, Heilongjiang, 1-11.VI.2011, Li Jingke	Tuss 01	KC80 1109	KC80 1284	KC80 1177
T. setifer setifer	Japan: Nara-ken; Kamikitayama-mura; Mt. Wasamatayama; 5.VI.2005; Satoru Nu leg.	99	EF57 0365	KC80 1286	KC80 1176
T. perrieri	Madagascar: La Mandraka, 16.I.2007, Olivier Boilly	Tpr0 2	KC80 1098	KC80 1273	KC80 1223
T. perrieri	Madagascar: La Mandraka, 16.I.2007, Olivier Boilly	Tpr0 3	KC80 1099	KC80 1274	X

Trox (*Phoberus*)

T. horridus	South Africa: Western Cape Prov., Gansekraal, 05.IX.2008, S33.52562° E18.32054°; 20m, C. Deschodt	ThG k02	KC80 1083	KC80 1243	KC80 1164
T. horridus	South Africa: Western Cape Prov., Gansekraal, 05.IX.2008, S33.52562° E18.32054°; 20m, C. Deschodt	ThG k06	KC80 1084	KC80 1244	KC80 1163
T. brincki	Lesotho: Mokhotlong District, 19.X.2007, S29°31'6.60" E29°11'12.99", 3089m, WP. Strümpher	TbL0 2	KC80 1079	KC80 1241	KC80 1140
T. brincki	Lesotho: Mokhotlong District, 19.X.2007, S29°31'6.60" E29°11'12.99", 3089m, WP. Strümpher	TbL0 4	KC80 1080	KC80 1242	KC80 1141
T. nasutus	South Africa: Western Cape Prov., near Simonstown, 07.IX.2008, S34.22200° E18.41069°, 80m, C. Deschodt	TnS T01	KC80 1097	KC80 1270	KC80 1228
T. nasutus	South Africa: Western Cape Prov., near Simonstown, 07.IX.2008, S34.22200° E18.41069°, 80m, C. Deschodt	TnS T02	KC80 1096	KC80 1269	KC80 1229
T. capensis	South Africa: Western Cape Prov., Moordenaarskop, 940m, 15.XII.2007, S34.09809° E18.96465°, WP Strümpher	CMK 05	KC80 1095	KC80 1271	KC80 1224
T. capensis	South Africa: Western Cape Prov., Sneeukop, S34.03919° E18.99026°, 1300m, 18.XII.2007, WP. Strümpher	CSn 03	KC80 1094	KC80 1272	KC80 1225

T. natalensis	South Africa: KZN, Durban, 16.XI.2008, S29°54'56.13" E30°56'9.13", WP. Strümpher	TnD 01	KC80 1101	KC80 1263	KC80 1147
T. natalensis	South Africa: KZN, Durban, 16.XI.2008, S29°54'56.13" E30°56'9.13", WP. Strümpher	TnD 04	KC80 1100	KC80 1264	KC80 1146
T. quadricostatus	South Africa: KZN, Ndumu Game Reserve, 2002.11.01, J. Harrison	Tq01	KC80 1103	KC80 1261	KC80 1143
T. quadricostatus	South Africa: KZN, Ndumu Game Reserve, 2002.11.01, J. Harrison	Tq02	KC80 1102	KC80 1262	KC80 1144
T. mozalae	Mozambique: Maputo Elephant Reserve. 27.XI.2007, S26.51006° E32.91270°, WP. Strümpher	mme r01	KC80 1104	KC80 1265	KC80 1142
T. mozalae	Mozambique: Maputo Elephant Reserve. 27.XI.2007, S26.51006° E32.91270°, WP. Strümpher	mme r02	KC80 1105	KC80 1266	KC80 1145
T. rhyparoides	South Africa: Eastern Cape Prov. Baviaanskloof, Poortjies (Poort); U. Kryger; 22.X-8.XI.2003	70A	EF57 0372	KC80 1267	KC80 1226
T. rhyparoides	South Africa: KZN, Ngome , A. Frolov, 2003	54A	EF57 0402	KC80 1268	KC80 1227
T. montanus	Kenya: Aberdare N.P.; 30.X.2002-03.XI.2002; 3100m; Between Kiandongoro & Mutobio gates, V. Grebennikov	22A	EF57 0377	KC80 1238	KC80 1152
T. montanus	Kenya: Aberdare N.P.; 30.X.2002-03.XI.2002; 3100m; Between Kiandongoro & Mutobio gates, V. Grebennikov	22B	EF57 0371	KC80 1239	KC80 1153
T. fascicularis	South Africa: Western Cape Prov., Sneeukop, S34.03919° E18.99026°, 1300m, 18.XII.2007, WP. Strümpher	ffSn0 3	KC80 1087	KC80 1256	KC80 1156
T. fascicularis	South Africa: Western Cape Prov., Sneeukop, S34.03919° E18.99026°, 1300m, 18.XII.2007, WP. Strümpher	ffSn0 4	KC80 1088	KC80 1257	KC80 1154

Species	Locality	Code			
T. strigosus	South Africa: Western Cape Prov., S31.48519° E23.73770°, 19.I.2008, WP Strümpher	Tstrg 01	KC80 1086	KC80 1255	KC80 1157
T. strigosus	South Africa: Western Cape Prov., S31.48519° E23.73770°, 19.I.2008, WP Strümpher	Tstrg 02	KC80 1085	KC80 1254	KC80 1155
T. nanniscus	South Africa: Eastern Cape Prov., Grahamstown, 635m, S33°16'14.79" E26°28'46.03", 14-16.XI.2008, WP. Strümpher	Tnan 05	KC80 1093	KC80 1260	KC80 1230
T. nanniscus	South Africa: Eastern Cape Prov., Grahamstown, 635m, S33°16'14.79" E26°28'46.03", 14-16.XI.2008, WP. Strümpher	Tnan 06	KC80 1092	KC80 1259	KC80 1231
T. rudebecki	Lesotho: Sani Top, 2880m, 19.X.2007, 29°34'43.12"S 29°16'50.75"E, WP. Strümpher	RL01	KC80 1089	KC80 1258	KC80 1158
T. rudebecki	Lesotho: Sani Top, 2880m, 19.X.2007, 29°34'43.12"S 29°16'50.75"E, WP. Strümpher	RL25	KC80 1090	X	KB80 1159
T. arcuatus	South Africa: Western Cape Prov., Kweekkraal farm; 9km west of Riversdale; U. Kryger; 22.X-8.XI.2003	69	EF57 0395	KC80 1249	KC80 1148
T. consimilis	South Africa: Northern Cape Prov., S31°12'26.74" Calvinia district, E19°40'47.14", 838m, 29.VIII.2009, CH. Scholtz	Tcon 01	KC80 1091	KC80 1250	KC80 1149
T. talpa	South Africa: Western Cape Prov., Kweekkraal farm; 9km west of Riversdale; U. Kryger; 22.X-8.XI.2003	68	EF57 0400	KC80 1248	KC80 1135
T. talpa	South Africa: Freestate Prov., Ladybrand, 1700m, 24.X.2007, S29.14971° E27.40688°, WP. Strümpher	Tt02	KC80 1081	KC80 1247	KC80 1134
T. sulcatus	South Africa: Freestate Prov., Parys, 13.X.2003, C.H. Scholtz	62	EF57 0397	KC80 1237	KC80 1160

T. sulcatus	South Africa: Eastern Cape Prov., Willowmore; Timbi 2; U. Kryger; 22.X-8.XI.2003	66	EF57 0398	KC80 1235	KC80 1162
T. squamiger	South Africa: Freestate Prov., Ladybrand, 1841m, 30.X.2007, S29.14971° E27.40688°, WP. Strümpher	SqL0 8	KC80 1078	KC80 1252	KC80 1151
T. squamiger	South Africa: Freestate Prov., Ladybrand, 1841m, 30.X.2007, S29.14971° E27.40688°, WP. Strümpher	SqL0 2	KC80 1077	KC80 1251	KC80 1150
T. nama	Namibia; Boom River, canyon ca.10km of estuary; A. Frolov leg.; 30-31.III.2003; S27°55'28.3" E17°01'14.6"; 590m	25A	EF57 0403	KC80 1253	KC80 1139
T. caffer	South Africa: Northern Cape Prov., nr. Hanover, S30.99228° E24.50198°, 1396m, 10.III.2008. WP. Strümpher	Tcaf 01	KC80 1076	KC80 1240	KC80 1137
T. cyrtus	South Africa: Northern Cape Prov., Kamieskroon, A. Frolov, C. Deschodt, 1-13.IX.2003; S30°15'58" E 17°55'30"	67B	EF57 0384	KC80 1236	KC80 1161
T. planicollis	South Africa: Northern Cape Prov., S31°12'26.74" E19°40'47.14", 29.VIII.2009. CH. Scholtz	Tpla n01	KC80 1075	KC80 1246	KC80 1138
T. luridus	South Africa: Western Cape Prov., Vanrhynsdorp, S31°32'0" E18°42'12", 05.VIII.2008, CH. Scholtz & WP Strümpher	Tlur0 1	KC80 1082	KC80 1245	KC80 1136

Table S2. Summary of oligonucleotide primers used in this study.

Gene	Primer	Direction	Primer sequence (5'→3')	Reference
16S	16sf (luisa)	forward	atgtctttttgakwataatwtaaag	Orsini *et al.* (2007)
	16sr (luisa)	reverse	acgctgttatccctaaggtaattt	Orsini *et al.* (2007)
18S	18s-intfw-st12	forward	atcaagaacgaaagttagag	Haring & Aspöck (2004)
	18s-rev1	reverse	atggggaacaattgcaagc	Haring & Aspöck (2004)
28S(D2)	D2-3551	forward	cgtgttgcttgatagtgcagc	Gillespie *et al.* (2005)
	D2-4057	reverse	tcaagacgggtcctgaaagt	Gillespie *et al.* (2005)
	Wer-F1	forward	taagygggtggtaaactc	This study
	Wer-R1	reverse	tatagcgtcgcygacgggcg	This study

Table S3. PCR thermal cycling profiles.

Gene	Stage 1 Initial denaturation	Stage 2 – Thermal cycling # Cycles	Denaturation	Annealing	Elongation	Stage 3 Final elongation
16S	94°C-90sec	35	(94°C-60sec,	48°C-90sec,	72°C-90sec)	72°C-1min
18S	95°C-2min	30	(95°C-10sec,	48°C-10sec,	72°C-90sec)	72°C-5min
28S(D2)	96°C-20sec	30	(96°C-15sec,	60°C-20sec,	72°C-60sec)	72°C-1min

Table S4. Data characteristics and estimated model parameters for 16S, 28S domain 2, 18S and

	16S	28S D2	18S	Combined
Number of samples	107	100	101	107
Aligned positions	405	601	562	1568
Paired characters (%)	112 (27.7)	268 (46.6)	350 (62.3)	730 (46.6)
Unpaired characters (%)	293 (72.3)	333 (55.4)	212 (37.7)	838 (53.4)
Parsimony informative sites	168	215	38	421
Tree length (MP)	918	676*	79	1779
Number of tree (MP)	10951	889*	208147	49
CI/RI (MP)	0.300/0.770	0.491/0.862*	0.620/0.942	0.381/0.840
Best fit model (AIC)	GTR+G	GTR+G	K80+I+G	GTR+G
A frequency	0.3555	0.2083	0.2134	0.7826
C frequency	0.814	0.3105	0.2622	0.2265
G frequency	0.1346	0.3144	0.3037	0.2555
T frequency	0.4284	0.1669	0.2206	0.2635
Gamma (G)	0.2040	0.2610	0.1460	0.1500
Invariable sites (I)	0	0	0.5600	0

CI = consistency index, RI = retention index, MP = Maximum Parsimony, * = MP analysis performed with reduced data set due to time and computational constraints. combined dataset.