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Transcriptomics illuminate the phylogenetic backbone of tiger beetles

HARLAN M. GOUGH[1,2*], JULIE M. ALLEN[3], EMMANUEL F. A. TOUSSAINT[4], CAROLINE G. STORER[1] and AKITO Y. KAWAHARA[1]

[1]*Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA*
[2]*Department of Biology, University of Florida, Gainesville, FL 32611, USA*
[3]*Department of Biology, University of Nevada, Reno, Reno, NV 89557, USA*
[4]*Natural History Museum of Geneva, CP 6434, CH 1211 Geneva 6, Switzerland*

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Phylogenomics is progressing rapidly, allowing large strides forward into our understanding of the tree of life. In this study, we generated transcriptomes from ethanol-preserved specimens of 13 tiger beetle species (Coleoptera: Cicindelinae) and one Scaritinae outgroup. From these 14 transcriptomes and seven publicly available transcriptomes, we recovered an average of 2538 loci for phylogenetic analysis. We constructed an evolutionary tree of tiger beetles to examine deep-level relationships and examined the extent to which the composition of the dataset, missing data, gene tree inconsistency and codon position saturation impacted phylogenetic accuracy. Ethanol-preserved specimens yielded similar numbers of loci to specimens originally preserved in costly reagents, showcasing more flexibility in transcriptomics than anticipated. The number of loci and gene tree inconsistency had less impact on downstream results than third codon position saturation and missing data. Our results recovered tiger beetles as sister to Carabidae with strong support, confirming their taxonomic status as an independent family within Adephaga. Within tiger beetles, phylogenetic relationships were robust across all nodes. This new phylogenomic backbone represents a useful framework for future endeavours in tiger beetle systematics and serves as a starting point for the development of less costly target capture toolkits to expand the taxonomic breadth of the future tiger beetle tree of life.

ADDITIONAL KEYWORDS: aTRAM – beetle phylogenomics – Carabidae – Cicindelidae – codon position saturation – RNA preservation – tiger beetle evolution.

INTRODUCTION

Molecular phylogenetics has become a key tool to understand the evolutionary history of life (Doolittle, 1999; Yang & Rannala, 2012). Even with a limited sampling of loci, we are often able to improve phylogenetic hypotheses. However, the power of inferences relying on molecular data is limited by the number of phylogenetically informative sites being examined (Edwards, 2009). Recently, transcriptome sequencing has been used to generate thousands of loci, allowing researchers to uncover old recalcitrant divergences in Eukaryotes (Riesgo *et al.*, 2012; Wen *et al.*, 2013; Kawahara & Breinholt, 2014; Misof *et al.*, 2014; Cunha & Giribet, 2019). Transcriptomic data can be analysed in conjunction with previously sequenced transcriptomes/genomes and, importantly, these transcriptomes can be used to develop target capture probe sets for future sequencing (Lemmon *et al.*, 2012; Pfeiffer *et al.*, 2019). A major hurdle to transcriptome sequencing is the rapid rate of degradation inherent to RNA (Sambrook *et al.*, 1989). Although best practice for preservation of RNA is usually to flash freeze specimens and maintain them at –80 °C until extraction, this is often impossible in the field (Sambrook *et al.*, 1989; Gorokhova, 2005). Some stabilization solutions, such as RNAlater, have been shown to be effective at preserving RNA at ambient temperatures (Mutter *et al.*, 2004), but it can be impossible to re-collect important specimens previously collected using different methods. New advances need to be made to allow the recovery of good-quality RNA for sequencing and, in turn, allow

*Corresponding author. E-mail: goughh@ufl.edu

the investigation of the evolution of groups for which phylogenomic hypotheses are still lacking.

One such group is the tiger beetles (Coleoptera: Carabidae: Cicindelidae), a clade comprising ~2900 named species found on all continents except Antarctica (Pearson & Vogler, 2001). United by several morphological synapomorphies, which include an expanded labrum extending laterally beyond the dorsomedial antennal insertions and long, curved mandibles possessing multiple teeth, tiger beetles have long been recognized as being monophyletic (Cassola, 2001). Despite their unique morphology, there remains debate about whether the group deserves family status or should be treated as a subfamily of Carabidae (Horn, 1915; Mandl, 1971; Cassola, 2001; López-López & Vogler, 2017; Gough *et al.*, 2019). In the era of traditional Sanger sequencing, molecular evidence has largely supported the subfamilial hypothesis (Maddison & Ober, 1999; McKenna *et al.*, 2015; Gough *et al.*, 2019), although there have been results suggesting that tiger beetles might instead be sister to Carabidae, thereby supporting the familial hypothesis (Bocak *et al.*, 2014). Maddision et al. (2009) found support for both topologies, with the ribosomal genes 28S and 18S supporting the subfamilial hypothesis and the nuclear gene *wingless* supporting the familial hypothesis. More recently, López-López & Vogler (2017) used mitochondrial genomes and the 18S nuclear ribosomal gene to argue that the placement of tiger beetles in a clade with three other carabid subfamilies (Scaritinae, Paussinae and Rhysodinae) was attributable to biased signal from the hypervariable regions of 18S. This assertion was strengthened by Zhang *et al.* (2018b), who found tiger beetles sister to the rest of Carabidae using 95 nuclear protein-coding loci. These two approaches represent a new link between Sanger sequencing and next-generation sequencing methods, but still lack the power to tackle the deep-scale phylogenomic relationships within tiger beetles properly.

Within tiger beetles, tribal classification has been unstable for centuries but has recently been reappraised with the advent of molecular phylogenetic hypotheses. Originally, Horn (1915) divided tiger beetles into four tribes (Manticorini, Megacephalini, Cicindelini and Collyridini) based on morphology. Based on a comprehensive morphological assessment, Rivalier (1950, 1954, 1957, 1961, 1969) retained these four tribes when he revised the classification of tiger beetles. The most recent world catalogue of tiger beetles by Wiesner (1992) recognizes the same four tribes plus Ctenostomini, but does not include more recent evidence brought by molecular studies, which were published a few years later.

The first molecular tribe-level phylogenies of tiger beetles questioned the monophyly of almost all tribes and recovered inconsistent relationships among them (Vogler & Pearson, 1996; Vogler & Barraclough, 1998; Galián *et al.*, 2002; Fig. 1). The previous study by Gough *et al.* (2019) represents the most comprehensive molecular phylogeny to date, relying on nine gene fragments, and is a good summary of the evidence that early molecular studies provided to understand the evolutionary relationships among tiger beetles. In that study, the tribe Platychilini (i.e. comprising the genera *Amblycheila*, *Omus*, *Picnochile* and *Platychile*) was recognized as paraphyletic owing to the inclusion of Manticorini. This clade, Manticorini + Platychilini, was robustly inferred as sister to the remainder of tiger beetles. The tribe Megacephalini was recovered as paraphyletic, with the genus *Omus* being included in Platychilini, and the genera *Cheiloxya*, *Oxycheila* and *Pseudoxycheila* forming a different and more derived clade corresponding to the subtribe Oxycheilina. This latter clade has been recovered consistently as sister to Cicindelini but has yet to be moved to a new official taxonomic designation (Vogler & Pearson, 1996; Vogler & Barraclough, 1998; Galián *et al.*, 2002; Gough *et al.*, 2019). The results from Gough *et al.* (2019) regarding the polyphyly of Megacephalini are in line with most previous molecular phylogenies (Vogler & Pearson, 1996; Vogler & Barraclough, 1998; Galián *et al.*, 2002). Even with this reorganization there is still disagreement about the placement of clades belonging to Megacephalini, with López-López & Vogler (2017) finding a different placement for Megacephalina as sister to Cicindelini with strong nodal support (Fig. 1). Gough *et al.* (2019) recovered the tribe Collyridini as paraphyletic, in line with some studies (Vogler & Pearson, 1996; Gough *et al.*, 2019) and in contrast to others (Vogler & Barraclough, 1998; Galián *et al.*, 2002). In contrast, the tribe Cicindelini appeared consistently to be monophyletic (Vogler & Pearson, 1996; Vogler & Barraclough, 1998; Galián *et al.*, 2002; López-López & Vogler, 2017; Gough *et al.*, 2019).

The discrepancies between these molecular studies indicate the difficulty in reconstructing old divergences using a limited number of loci (Fig. 1). In order to gain a better understanding of the placement of tiger beetles in Adephaga and to establish a tribe-level backbone phylogeny for tiger beetles, we sequenced transcriptomes from four out of five recognized tiger beetle tribes (Cicindelini, Collyridini, Manticorini, Megacephalini and Platychilini) and used a greatly expanded set of loci to infer a robust backbone tree within this charismatic group of beetles.

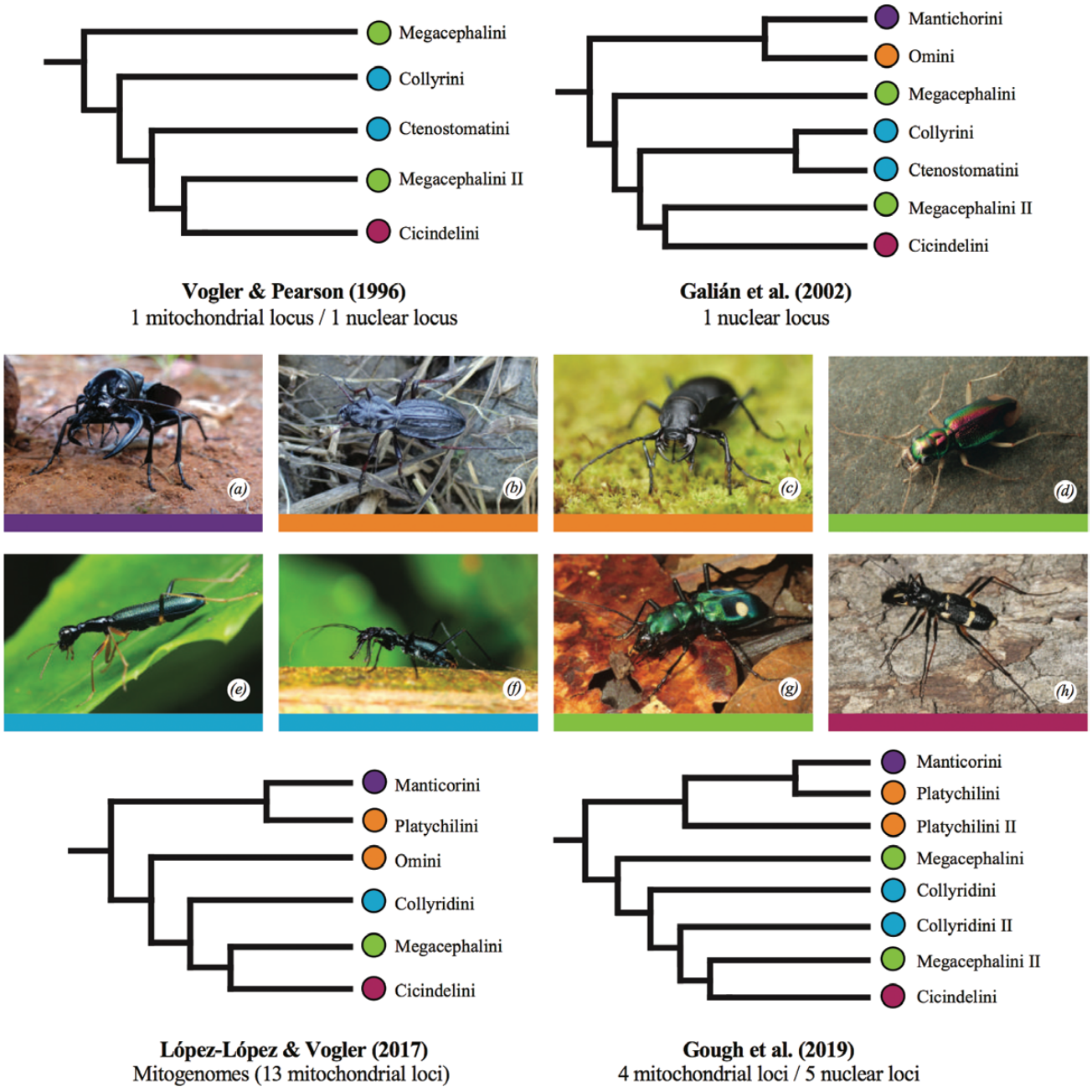

Figure 1. Previous tribe-level hypotheses of phylogenetic relationships among tiger beetles. All tribal names correspond to the original labelling of the different studies listed below. A, phylogenetic hypothesis inferred by Vogler & Pearson (1996) based on the combined analysis of the mitochondrial 16S gene and nuclear 18S gene (including hypervariable regions). B, phylogenetic hypothesis inferred by Galián *et al.* (2002) based on the analysis of the 18S gene (including hypervariable regions). C, from top to bottom and from left to right: (a) *Manticora scabra* (credit: Bernard Dupont); (b) *Picnochile fallaciosa* (credit: Yasuoki Takami); (c) *Omus californicus* (credit: Ken Hickman); (d) *Tetracha carolina* (credit: David Maddison); (e) *Neocollyris* sp. (credit: Tyus Ma); (f) *Pogonostoma* sp. (credit: Michel Candel); (g) *Pseudoxycheila* sp (credit: Andreas Kay); and (h) *Distipsidera flavipes* (credit: Malcolm Tattersall). D, phylogenetic hypothesis inferred by López-López & Vogler (2017) based on the combined analysis of mitogenomes and nuclear 18S gene (including or excluding hypervariable regions). E, phylogenetic hypothesis inferred by Gough *et al.* (2019) based on a supermatrix approach using four mitochondrial genes and five nuclear genes.

MATERIAL AND METHODS

Taxon sampling

We collected 13 tiger beetle species from across tiger beetle main lineages and one outgroup from the subfamily Scaritinae. Thirteen specimens were collected into 96% biological grade ethanol in the field and one was flash frozen using liquid nitrogen (Table 1). All specimens were transferred to freezers at −80 °C for long-term storage, but the ethanol specimens differed in the conditions to which they were subjected before long-term storage. Three specimens were transferred to −80 °C within 1 day of collection, five specimens were stored at ambient temperatures for 10–14 days before transfer, and six were stored at −20 °C for 2–3 months before being transferred to −80 °C (Table 1). In order to test the placement of tiger beetles within Adephaga, we also included seven outgroups representing different subfamilies of Carabidae and families within Adephaga (Supporting Information, Table 1). The seven transcriptomes were downloaded from the GenBank sequence read archive (SRA).

RNA extraction and sequencing

RNA extractions were performed on macerated thoracic muscle using an OmniPrep micro RNA kit (G-Biosciences, St Louis, MO, USA) and quantified using a Qubit RNA HS Assay kit according to the manufacturer's instructions. RNA extracts were subsequently inspected for quality and degradation using an Agilent 2200 Tape Station Total RNA assay at the University of Florida Interdisciplinary Center for Biotechnology (UF ICBR; Gainesville, FL, USA). Extracts were prepared for sequencing using the Illumina TruSeq RNA Library Prep Kit, following the Low Sample protocol using 1 μg of RNA and indexes from TruSeq kits A and B. Indexes were assigned according to the manufacturer's pooling guidelines. Libraries were then quantified using the Qubit DNA BR Assay kit, run on a 2% agarose gel, normalized to 10 nM, and 10 μL of each normalized library was pooled. Evaluation of the pooled library and quality testing were performed before Illumina HiSeq3000 2 × 100 paired end sequencing at the UF ICBR. The seven additional transcriptomes were added before the bioinformatic pipeline to process all data together. The raw reads of all 21 transcriptomes were trimmed and filtered by quality using TrimGalore! (www.bioinformatics.babraham.ac.uk/projects/trim_galore).

Locus assembly

In order to identify target loci for phylogenetic analysis, we created a reference locus set from the genome of *Tribolium castaneum* (Tribolium Genome Sequencing Consortium, 2008). Using OrthoDB v.9.1 (Zdobnov *et al.*, 2017), we selected loci represented in the six currently available beetle genomes (Keeling *et al.*, 2013; McKenna *et al.*, 2016; Schoville *et al.*, 2018). None of the six genomes available was from the suborder Adegephaga, to which tiger beetles belong; therefore, we were cognizant that paralogues could be present between the reference genomes and our study taxa. To limit the number of potential paralogues in our dataset, we selected only loci identified as single copy within all six published genomes. Our final reference set of loci consisted of 4225 loci.

Transcriptome assembly and searches for the target loci were performed using the program aTRAM v.2.0 (Allen *et al.*, 2018). This program allows for genomic data assembly against a set of specified references. For each locus, aTRAM first blasts the target locus against the unassembled reads of a single transcriptome. Matching hits and their mate pairs are assembled *de novo*. The assembled contigs are then blasted back against the unassembled transcriptome reads, and again the matching hits and their mate pairs are assembled *de novo*, and this iterative process is repeated. We used the amino acid sequences from 4225 loci from *Tribolium castaneum* as the reference in aTRAM, with the assembler TRINITY v.2.5.1 (Grabherr *et al.*, 2011) and five iterations to build contigs for each locus from all 21 unassembled transcriptomes. The assembled contigs were processed using the exon-stitching pipeline, returning a single fasta file for each locus containing only exon sequence from every taxon (Allen *et al.*, 2017). After assembly for all 21 transcriptomes, 3995 loci were retained.

Post aTRAM cleaning and alignment

A reciprocal best blast was performed between all the assembled contigs and the entire *Tribolium castaneum* proteome. Any contigs that had best hits to loci that were not the target locus used in their assembly were removed as described by Allen *et al.* (2017). After the reciprocal best blast, 3735 loci were retained. Loci were filtered by the number of taxa captured, and three datasets were created to examine the effects of locus selection on phylogenetic reconstruction. The first dataset contained loci with 100% taxon occupancy, the second dataset contained loci with a minimum of 90% taxon occupancy, and the last dataset contained only loci with a minimum of 75% taxon occupancy. All three datasets were aligned and translated using PRANK (Löytynoja, 2014), resulting in six datasets, one nucleotide and one amino acid dataset for each of the three different taxonomic occupancy cut-offs (Table 1). To remove

Table 1. Taxon sampling and RNA extraction results

Genus	Species	Accession no.	Preservation	Conditions and time before storage at −80 °C	RNA (ng/μL)	Loci recovered (*N*)	Loci recovered (%)
Ellipsoptera	*hamata*	(Lib01)	96% ethanol	1 day at ambient temperature	47.8	2705	64.02
Tetracha	*carolina*	(Lib2)	Frozen in liquid nitrogen	Directly into −80 °C	55	2832	67.03
Apterodela	*unipunctata*	(Lib03)	96% ethanol	< 1 day at ambient temperature	32.2	2899	68.62
Oxycheila	*nigroaenea*	(Lib04)	96% ethanol	10 days at ambient temperature	114	2917	69.04
Ctenostoma	*arnaudi*	(Lib05)	96% ethanol	10 days at ambient temperature	49	2859	67.67
Dromica	*spectabilis*	(Lib06)	96% ethanol	14 days at ambient temperature	8.64	2606	61.68
Odontocheila	sp.	(Lib07)	96% ethanol	10 days at ambient temperature	13.4	2312	54.72
Cicindela	*pulchra*	(Lib08)	96% ethanol	25 days at 5 °C, ~2 months at −20 °C	104	2625	62.13
Cicindelidia	*obsoleta*	(Lib09)	96% ethanol	24 days at 5 °C, ~2 months at −20 °C	84	2522	59.69
Calomera	*littoralis*	(Lib10)	96% ethanol	13 days at ambient temperature, ~3 months at −20 °C	24.4	2445	57.87
Habroscelimorpha	*dorsalis*	(Lib11)	96% ethanol	< 1 day at ambient temperature	96	2562	60.64
Eunota	*fulgoris*	(Lib12)	96% ethanol	12 days at 5 °C, ~2 months at −20 °C	87	2807	66.44
Amblycheila	*cylindriformis*	(Lib13)	96% ethanol	4 days at 5 °C, ~2 months at −20 °C	45.6	2695	63.79
Pasimachus	*viridans*	(Lib14)	96% ethanol	22 days at 5 °C, ~2 months at −20 °C	212	2830	66.98
Carabus	*granulatus*	(SRR596983)	RNAlater	Unknown	Unknown	2154	50.98
Paussus	*spinicoxis*	(SRR1727931)	Unknown	Unknown	Unknown	1753	41.49
Pogonus	*chalceus*	(SRR424344)	Frozen in liquid nitrogen	Unknown	Unknown	3344	79.15
Metrius	*contractus*	(SRR1727930)	Unknown	Unknown	Unknown	1769	41.87
Gyrinus	*marinus*	(SRR921604)	RNAlater	Unknown	Unknown	2843	67.29
Amphizoa	*insolens*	(SRR5930489)	Unknown	Unknown	Unknown	2205	52.19
Priacma	*serrata*	(SRR596769)	RNAlater	Unknown	Unknown	1625	38.46

non-parsimony-informative sites, the program trimAl (Capella-Gutiérrez *et al.*, 2009) was used to remove all columns containing fewer than three taxa.

The six datasets were analysed in two different ways to test potential locus tree inconsistency. First, the datasets were concatenated, using FASconCAT-G v.1.02 (Kück & Longo, 2014), and analysed using maximum likelihood. Second, the datasets were analysed using coalescent-based species tree estimation, where each locus was used to infer an individual locus tree. Finally, from the 100% taxon occupancy concatenated nucleotide dataset, we generated two more datasets, one that included only the third codon positions of all loci and the other that included only the first and second codon positions of all loci. These two datasets were analysed following the same methods as the other concatenated nucleotide datasets (Appendix S2). We also explored third codon position saturation in our datasets using DAMBE7 (Xia, 2018).

Data partitioning, model selection and phylogenetic analyses

Partitioning schemes were searched using PartitionFinder v.2.1.1 (Lanfear *et al.*, 2017) with the relaxed clustering algorithm (rcluster) and default settings (i.e. -rcluster 10) and using the Bayesian information criterion (BIC) to select the optimal models of nucleotide and amino acid substitutions. Owing to the size of the datasets, we adopted a two-step approach, in which partitions were initially estimated based on a reduced model set of commonly used models, then models of substitution for each partition were estimated subsequently using all available models in IQ-TREE. To do so, the number of models tested in PartitionFinder was limited to GTR, GTR+G and GTR+I+G for nucleotide datasets or WAG, WAG+G and WAG+GAU+F for amino acid datasets. For the largest datasets (75% taxon occupancy), the number of models was restricted further to GTR for nucleotide datasets and WAG for amino acid datasets and, additionally, the rcluster algorithm was set to examine only the top 5% of partitioning schemes (i.e. -rcluster 5). ModelFinder (Kalyaanamoorthy *et al.*, 2017) in IQ-TREE v.1.6.9 (Nguyen *et al.*, 2015) was used to determine the correct model for each partition identified using PartitionFinder across all available models. The resulting partitioning schemes and models for each partition were used as inputs for the maximum likelihood phylogenetic analyses conducted in IQ-TREE. We performed 100 independent tree searches on all datasets, each with 1000 ultrafast bootstraps (UFBoot) (Minh *et al.*, 2013; Hoang *et al.*, 2018), and 1000 SH-aLRT tests (Guindon *et al.*, 2010), which are a modified approximate likelihood ratio test, to investigate the nodal support across the topology. To reduce the risk of overestimating branch support with UFBoot when the model incorrectly estimated rate heterogeneity among sites, we used hill-climbing nearest neighbour interchange (NNI) to optimize each bootstrap tree (Appendix S3).

To investigate possible discordant signals between loci, we produced locus trees using IQ-TREE. The loci were not partitioned, and models of nucleotide substitution were determined in IQ-TREE using the BIC. We estimated species trees for each dataset from the locus trees using ASTRAL v.5.6.2 (Zhang *et al.*, 2018a) (Appendix S4). All analyses were performed on the HiPerGator v.2.0 cluster at the University of Florida.

RESULTS AND DISCUSSION

Recovery of loci

Of the 3735 loci retained after the reciprocal best blast, we were able to recover, on average, 2538 loci per taxon (Table 1). This recovery rate is surprising, because the target loci sequences from *Tribolium castaneum*, the reference taxon, are likely to be between 280 and 330 Myr divergent from the taxa we sequenced (Hunt *et al.*, 2007; Toussaint *et al.*, 2017; Zhang *et al.*, 2018b). The three different taxon occupancy cut-offs of 75, 90 and 100% yielded 2125, 1280 and 202 loci, respectively (Supporting Information, Appendix S1). Missing data accounted for 37% of the 75% taxon occupancy dataset, 35% of the 90% occupancy dataset and 25% of the 100% taxon occupancy dataset. Transcriptomes pulled from GenBank SRA captured 53% of loci, on average, whereas the newly sequenced transcriptomes captured 64% of loci, on average. Preservation and storage did not have a significant effect on the number of loci captured in our study. The specimen flash frozen in liquid nitrogen captured 67% of loci. The three specimens transferred to the freezers at –80 °C within 1 day of collection captured 64% of loci, on average; the five specimens stored at ambient temperatures for 10–14 days before transfer captured 63% of loci, and the six specimens stored at –20 °C for 2–3 months before being transferred to the freezers at –80 °C captured 63% (Table 1).

Our results show that sufficient RNA for conducting phylogenetic transcriptomics was preserved in specimens collected into 96% ethanol even when these specimens were not transferred immediately to –80 °C freezers and confirm previous studies showing substantial RNA preservation in ethanol (Bazinet *et al.*, 2013; Astrid *et al.*, 2016). This is an important result for empirical phylogenomic/transcriptomic studies of non-model organisms, because solutions designed to preserve RNA are often very expensive, and flash freezing in liquid nitrogen is usually restricted

to local studies near research facilities. Our study provides strong evidence that specimens preserved in 96% biological grade ethanol can be used for RNA sequencing and transcriptome assembly regardless of their storage conditions. Although more replicates are needed to test the resilience of RNA to storage in ethanol, we predict that this encouraging result will be of great interest to the community, because it opens a new window into the study of organisms for which complex RNA storage procedures are often not logistically practical and/or affordable.

PHYLOGENOMICS OF TIGER BEETLES

The phylogenetic hypotheses for tiger beetles inferred with IQ-TREE and ASTRAL using nucleotide datasets of all three taxon occupancies (100, 90 and 75%) were identical (Fig. 2). Analyses of all these datasets recovered tiger beetles as sister to Carabidae with strong support (Table 2). This result supports the conclusion that a signal from 18S in Sanger sequencing studies of the group might be responsible for the incorrect placement of tiger beetles as deeply nested within Carabidae and that tiger beetles can

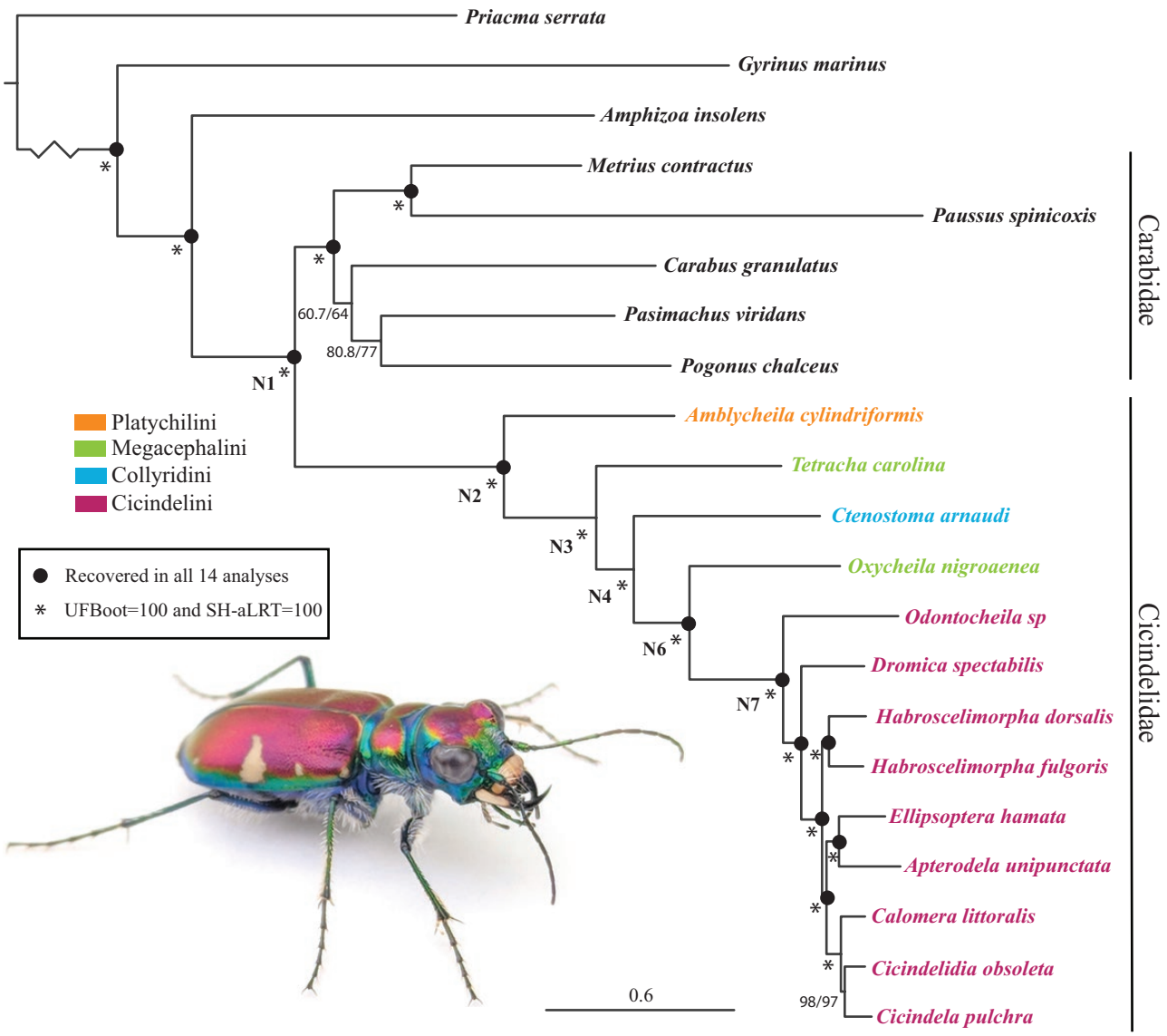

Figure 2. Maximum likelihood phylogenetic hypothesis based on the 100% taxon occupancy amino acid dataset. The branch between the outgroup *Priacma serrata* and the ingroup taxa was broken to condense the figure. *UFBoot = 100 and SH-aLRT = 100. Black circles represent nodes that were recovered in all 14 analyses. A photograph of *Cicindela pulchra* is presented (credit: Harlan Gough).

be considered an independent family (López-López & Vogler, 2017; Zhang *et al.*, 2018b). The three amino acid datasets showed nearly identical relationships between the 13 sampled tiger beetles.

Tribal relationships were consistent between the nucleotide and amino acid datasets except for a single node. Amino acid datasets recovered all tribes as a phylogenetic grade, with Platychilini sister to all remaining tiger beetles, whereas nucleotide datasets recovered the same phylogenetic grade, but with Megacephalini and Collyridini as sister clades (Table 2). The topology recovered in the analysis of the first and second codon positions matched that of the amino acid datasets, and the analysis of the third codon position matched that of the nucleotide analysis. This suggests that a signal from the third codon position is likely to be driving the placement of Megacephalini and Collyridini in our nucleotide datasets. Saturation plots, produced in DAMBE, of model-corrected genetic distance by codon position plotted against transitions and transversions indicate that the third codon position was partly saturated, suggesting that the signal coming from the nucleotide datasets might be biased (Fig. 3). Studies have explored issues with saturation in phylogenomic datasets and have found that saturated third codon positions can negatively influence phylogenetic

Table 2. Nodal support for all 14 phylogenetic analyses

	Node 1	Node 2	Node 3	Node 4	Node 5	Node 6	Node 7
A1	100/100	100/100	100/100	100/100	NR	100/100	100/100
A2	1	1	1	1	NR	1	1
A3	100/100	100/100	100/100	100/100	NR	100/100	100/100
A4	1	1	1	1	NR	1	1
A5	100/100	100/100	100/100	100/100	NR	100/100	100/100
A6	1	1	1	1	NR	1	1
A7	100/100	100/100	NR	NR	100/100	100/100	100/100
A8	1	1	NR	NR	1	1	1
A9	100/100	100/100	NR	NR	100/100	100/100	100/100
A10	1	1	NR	NR	1	1	1
A11	100/100	100/100	NR	NR	52.9/57	100/100	100/100
A12	1	1	NR	NR	0.99	1	1
A13	100/100	100/100	NR	NR	77.7/86	100/100	100/100
A14	100/100	100/100	100/100	100/100	NR	100/100	100/100

Support values for maximum likelihood analyses are given as UFBoot/SH-aLRT, whereas support for ASTRAL analyses are given as local posterior probabilities. Nodes are labelled in Figure 3, except for node 5, which was not recovered in the displayed analysis. Node 5 represents a clade formed by *Tetracha carolina* and *Ctenostoma arnaudi*.
Abbreviations: A1, amino acid, 75% taxon occupancy, IQ-TREE; A2, amino acid, 75% taxon occupancy, ASTRAL; A3, amino acid, 90% taxon occupancy, IQ-TREE; A4, amino acid, 90% taxon occupancy, ASTRAL; A5, amino acid, 100% taxon occupancy, IQ-TREE; A6, amino acid, 100% taxon occupancy, ASTRAL; A7, nucleotide, 75% taxon occupancy, IQ-TREE; A8, nucleotide, 75% taxon occupancy, ASTRAL; A9, nucleotide, 90% taxon occupancy, IQ-TREE; A10, nucleotide, 90% taxon occupancy, ASTRAL; A11, nucleotide, 100% taxon occupancy, IQ-TREE; A12, nucleotide, 100% taxon occupancy, ASTRAL; A13, nucleotide, 100% taxon occupancy, IQ-TREE, first and second codons; A14, nucleotide, 100% taxon occupancy, IQ-TREE, third codon; NR, node not recovered.

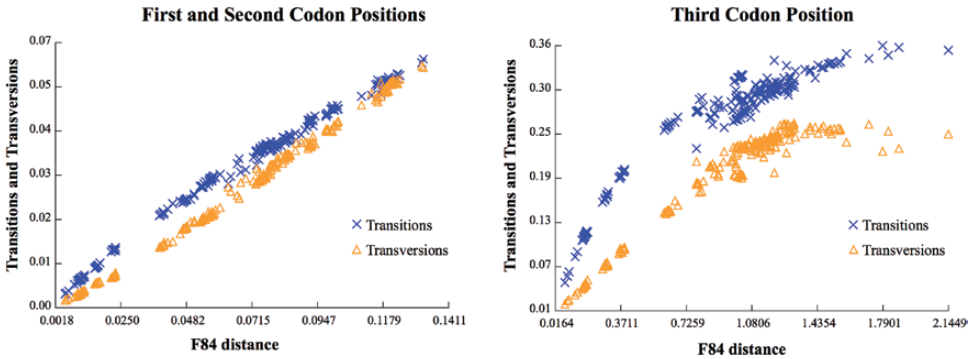

Figure 3. DAMBE saturation plot of model-corrected genetic distance by codon position plotted against transitions and transversions. In the graph of third codon positions, the levelling off of transitions as the site-wise F84 genetic distance increases is indicative of saturation. This same levelling off is not apparent in the graph of first and second codon positions.

reconstruction, especially at deeper nodes (Breinholt & Kawahara, 2013; Rota-Stabelli *et al.*, 2013; Cox *et al.*, 2014). For this reason, we prefer the topology resulting from analyses of the amino acid datasets and from the analysis of only the first and second codon positions of the nucleotide dataset.

The topology inferred in this study (Fig. 2) corroborates the results of Gough *et al.* (2019) but with higher bootstrap support overall. The placements of Collyridini and Megacephalini differ from those in the tree of López-López & Vogler (2017; Fig. 2). This suggests that the hypervariable region of 18S does not negatively affect tree estimation for tiger beetles at the tribal level in the same way it does the placement of tiger beetles relative to Carabidae. The placement of the genus *Oxycheila* as sister to the rest of Cicindelini is consistent with many previous studies and renders the tribe Megacephalini polyphyletic (Vogler & Pearson, 1996; Galián *et al.*, 2002; Gough *et al.*, 2019). Although our taxon sampling is not sufficient to test the monophyly of all tribes of tiger beetles, we are able to establish a robust tribal backbone, which had previously only been estimated based on a few mitochondrial and nuclear loci. Future efforts should be focused on sampling key lineages that potentially render some tribes paraphyletic.

At the genus level, our inferences are equally robust across datasets, with a unique area of topological conflict in the placement of *Calomera littoralis*. In the 75% taxon occupancy IQ-TREE analysis, *Calomera littoralis* is recovered as sister to *Cicindela pulchra* with low support (UFBoot = 57.1, SH-aLRT = 69), whereas in the 100 and 90% taxon occupancy amino acid datasets it is recovered as sister to both *Cicindelidia obsoleta* and *Cicindela pulchra* with strong support (UFBoot = 100, SH-aLRT = 100), with the latter relationship corroborating Gough *et al.* (2019). This topological conflict could be the result of the larger amount of missing data inherent to the 75% taxon occupancy dataset. The average percentage of missing data increases from 25% in the 202 loci dataset to 37% in the 75% taxon occupancy dataset. Interestingly, all ASTRAL amino acid dataset analyses including the analysis on the 75% taxon occupancy dataset recover the more common topology of *Calomera littoralis* as sister to both *Cicindelidia obsoleta* and *Cicindela pulchra* with strong support.

Increasing the number of loci affected the topology within tiger beetles only in this single instance and raised support values of six nodes only across these 12 analyses. This suggests that the 202 loci in the 100% taxon occupancy dataset are sufficient to determine tribe-level relationships within tiger beetles and recover more derived relationships with strong support. This result is consistent with other transcriptomic studies that found the same topology with differing numbers of loci and taxonomic occupancy (Lemer *et al.*, 2015; Fernández *et al.*, 2016). This set of loci will serve as a starting point to design a probe set to allow the capture of large genomic fractions for a much denser taxon sampling of tiger beetles.

Conclusions

Our study of newly generated transcriptomes yields a robust backbone for tiger beetle relationships and adds support for the classification of tiger beetles as their own family. Nodal support across all analyses is strong regardless of the phylogenetic analysis (concatenation vs. species tree estimation) and dataset type (nucleotide or amino acid). Our study provides evidence that specimens preserved in 96% ethanol can retain enough RNA for transcriptomic analysis. Importantly, the transcriptomes sequenced in this study will facilitate the construction of an anchored hybrid enrichment probe set which, owing to lower sequencing cost per specimen, will allow for a greatly expanded taxonomic sampling and a better understanding of the evolutionary history of tiger beetles.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Taxon sampling and loci captured for all transcriptomes.
AppendixS2. Matrices used in the maximum likelihood phylogenetic analyses done in IQ-TREE.
Appendix S3. Treefiles used in the maximum likelihood phylogenetic analyses done in IQ-TREE.
Appendix S4. Gene trees used in the ASTRAL analyses.

SHARED DATA

Raw and processed transcriptomes are available on Dryad: https://doi.org/10.5061/dryad.8w9ghx3h9 (Gough *et al*., 2020).