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TECHNICAL NOTE

DNA-based identification of preys from non-destructive, total DNA extractions of predators using arthropod universal primers

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Abstract

Here, I show that prey sequences can be detected from DNA of tiger beetles of the genus *Rivacindela* using whole specimens, nondestructive methods, and universal cytochrome b primers for arthropods. BLAST searches of the obtained sequences against public databases revealed that the diet of *Rivacindela* is mostly composed of flies but also termites and other beetles. Accurate determination of order, family and even genus was achieved in most cases but rarely to species level. Results suggest that stored DNA samples extracted from whole predatory specimens could be an alternative to dissected gut contents as starting source for DNA-based dietary studies.

Keywords: cytochrome b, insects, PCR, predatory studies, *Rivacindela*, universal primers

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Our laboratory has recently studied a poorly known Australian radiation of predatory tiger beetles of the genus *Rivacindela* to test species delineation and DNA taxonomy using mitochondrial DNA (mtDNA) sequences of *coxI*, *rrnL* and *cob* genes (Pons *et al*., unpublished data). This survey included 468 specimens from 65 ephemeral lakes that were immediately preserved in ethanol after collection. DNA extraction was performed by non-destructive methods to conserve specimens for subsequent vouchering and morphological study. Thus whole specimens were air-dried, submerged in DNA extraction buffer overnight and removed before performing subsequent steps (QIAGEN DNeasy kit). *Cob* electropherograms of some 50 specimens showed elevated background noise and double peaks, and hence sequences were not included in that study. *Cob* polymerase chain reaction (PCR) products of seven specimens (S00–S06, Table 1) showed readable electropherograms, with elevated background but lacking double peaks. Sequences were about 20–32% divergent relative to any other *Rivacindela cob* sequences, which intrageneric divergence was lower than 15% (Pons *et al*., unpublished data). Surprisingly, the seven suspicious sequences neither revealed stop codons nor indels relative to other mitochondrial sequences (358 bp), i.e. represent orthologous sequences, not NUMTs (nuclear mitochondrial DNA sequences originated by transposition from mitochondrion to nucleus). The first beetle pseudogene was only reported recently, for some *rrn*L sequences from other *Rivacindela* species (Pons & Vogler 2005).

BLAST N search against sequences deposited in public databases (www.ncbi.nlm.nih.gov/) revealed that sequences of two specimens showed 100% identity with *cob* sequences of the hymenopteran *Apis melifera*, and the remaining five 85% similarity with *cob* sequences of the dipteran *Scathophaga tropicalis* (Table 1). Identical results were observed after repeating PCRs and sequence analyses. Since controls testing DNA extraction and PCR reagents were both negative, and because bees and dipterans have never been studied in our laboratory, we rejected an external contamination.

An alternative hypothesis could be the occurrence of parasites or prey residues inside guts in some *Rivacindela* specimens whose DNAs were coisolated with *Rivacindela* genomic DNA. These coextracted DNAs could also be amplified because PCRs were performed with universal arthropod oligonucleotides at low annealing temperature. *Cob* primers used here (CB3 5′-GAGGAGCAACTGTAATTACTAA-3′ and CB4 5′-AAAAGAAARTATCATTCAGGTTGAAT-3′; Barraclough *et al*. 1999) were successful in a

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Table 1 BLAST N results of the 30 *cob* sequences amplified in the 14 *Rivacindela* specimens studied here. Sequences were obtained directly from PCR products or from clones when double peaks were visible in direct sequencing. The first hit for each query sequence is included here although the following hits (not shown) also matched sequences with similar taxonomic status (order and family) and for most of them even genus. Sequences from *Rivacindela* specimens 1a.3, 70.2, 85.1, 114.4, and 121.3 were used as positive sequence queries, which showed 100% identity with the *cob* sequences deposited in GenBank for the same individuals

			BLAST results					
Rivacindela specimen	Sequence	PCR/no. of clones	Species	Accession no.	*E* value	% similarity	Order	Family
67a.2	S00	PCR	*Apis melifera*	U72276	0.0	100	Hymenoptera	Apidae
67a.3	S01	PCR	*Apis melifera*	U72276	0.0	100	Hymenoptera	Apidae
68a.1	S02	PCR	*Scathophaga tropicalis*	AF180982	6e-70	85	Diptera	Scathophagidae
68a.5	S03	PCR	*Scathophaga tropicalis*	AF180982	6e-70	85	Diptera	Scathophagidae
68a.8	S04	PCR	*Scathophaga tropicalis*	AF180982	6e-70	85	Diptera	Scathophagidae
68b.3	S05	PCR	*Scathophaga tropicalis*	AF180982	6e-70	85	Diptera	Scathophagidae
68c.1	S06	PCR	*Scathophaga tropicalis*	AF180982	6e-70	85	Diptera	Scathophagidae
140.7	S07	3	*Rivacindela JP32b*	AJ618481	5e-123	90	Coleoptera	Cicindelidae
68a.9	S08	1	*Cicindela fulgoris*	AF438944	1e-74	86	Coleoptera	Cicindeliade
	S09	4	*Rivacindela JP60.2*	AJ618586	1e-163	95	Coleoptera	Cicindeliade
91.6	S10	3	*Rivacindela JP97.4*	AJ618684	8e-125	91	Coleoptera	Cicindeliade
94a.3	S11	3	*Scathophaga incola*	AF180980	4e-77	85	Diptera	Scathophagidae
	S12	2	*Scathophaga obscura*	AF180984	6e-107	90	Diptera	Scathophagidae
	S13	2	*Asphondylia* sp.	AY277751	7e-85	87	Diptera	Cecidomyiidae
	S14	2	*Rivacindela JP90.5*	AJ618663	1e-138	92	Coleoptera	Cicindelidae
	S15	2	*Rivacindela JP94a.4*	AJ618677	5e-49	82	Coleoptera	Cicindelidae
	S16	2	*Armigeres subalbatus*	AY439851	8e-20	92	Diptera	Culicidae
	S17	1	*Lophyra catena*	AJ515078	2e-26	85	Coleoptera	Cicindelidae
140.5	S18	5	*Rivacindela JP202.1*	AJ618744	3e-161	95	Coleoptera	Cicindelidae
	S19	1	*Cicindela tranquebarica*	AF438938	0.0	98	Coleoptera	Cicindelidae
	S20	1	*Cryptotermes brevis*	AF189112	2e-26	82	Isoptera	Kalotermitidae
	S21	1	*Cicindela fulgoris*	AF438944	1e-74	86	Coleoptera	Cicindelidae
146.3	S22	4	*Blackburnia kukui*	AF534949	3e-50	84	Coleoptera	Carabidae
	S23	1	*Cicindela californica*	AF439126	1e-64	84	Coleoptera	Cicindelidae
	S24	6	*Rivacindela JP68b.2*	AF618616	5e-58	83	Coleoptera	Cicindelidae
146.7	S25	1	*Rivacindela JP201.1*	AJ618743	7e-42	84	Coleoptera	Cicindelidae
	S26	2	*Archaeochlus drakensbergensis*	AY263802	1e-77	86	Diptera	Chironomidae
	S27	6	*Blackburnia kukui*	AF534949	1e-52	84	Coleoptera	Carabidae
	S28	1	*Cicindela cazieri*	AF439109	0.0	98	Coleoptera	Cicindelidae
	S29	1	*Rivacindela JP97.4*	AF618684	8e-125	91	Coleoptera	Cicindelidae

wide range of arthropods such as cockroaches, termites, butterflies, moths, beetles and spiders (A. Vogler, personal communication). PCR started with an initial denaturation step at 94 °C for 3.5 min, followed by 40 cycles at 94 °C for 30 s, 46 °C for 30 s, and 72 °C for 1 min, and finally an extension step at 72 °C for 10 min. Evidence of alien DNA sequences was centred around most of the individuals of few closely related populations (67, 68, 91, 94, 140 and 146), and only observed with *cob* primers. These results suggest that *cob* sequences of those *Rivacindela* populations did not match universal primers and hence, in absence of competition, prey or parasite copies at lower copy number could be amplified. Perhaps it was only observed in *cob* marker because prey vestiges are partially degraded in guts, and under those circumstances, shorter sequences (*cob* 358 bp) are more feasible to obtain than longer ones (*cox*1 800 bp and *rrn*L 900 bp). The discovery that GenBank honeybee entry U72276 (unpublished) was obtained from Australian specimens would reinforce the hypothesis of prey vestiges in guts.

The aims of this study were: (i) show that universal arthropod *cob* primers detect 'alien' DNA in samples extracted from *Rivacindela* and (ii) characterize the alien sequences and determine their taxonomic status by comparing them against public databases. Altogether could shed light on which species are the preys of *Rivacindela* beetles, and show that DNA extracted from whole predatory specimens is an alternative source to gut contents in predatory analysis. We used a DNA-based approach similar to that employed in dietary studies of marine invertebrates

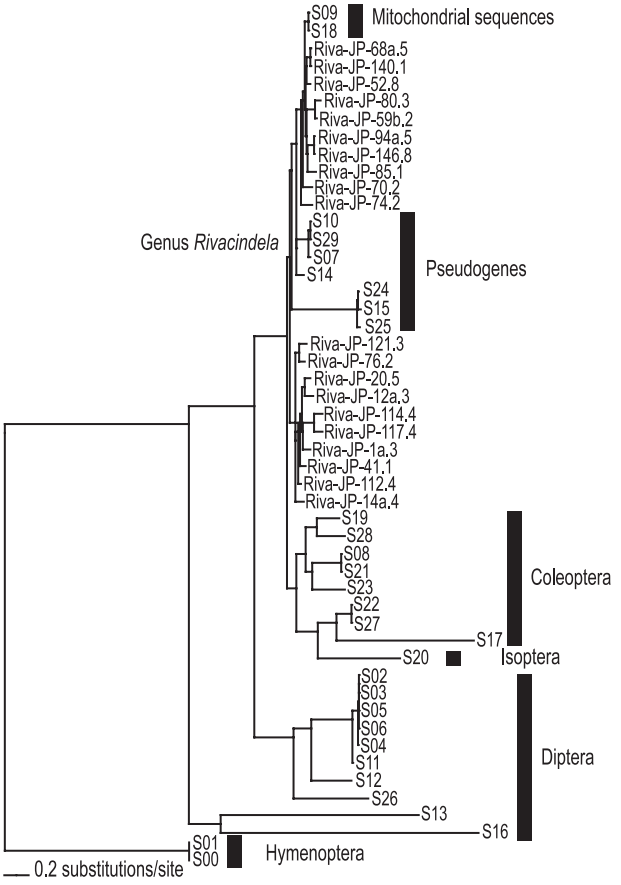

Fig. 1 Phylogram of the 30 different *cob* sequences (S00–S29, see Table 1) obtained here plus the mitochondrial sequences (Riva-JP) obtained in 20 *Rivacindela* specimens (Pons *et al*., unpublished data). Topology, parameters and branch lengths were estimated in the program PHYML 2.4.4 under the GTR+Γ+I model selected by MODELTEST. Taxonomic status of the sequences S00–S29 was estimated by BLAST N (first hit) and is indicated by vertical bars. Sequences S00–S18, S20–S27 and S29 were deposited in EMBL Nucleotide Sequence Database under the Accession nos AM159539–AM159566.

(Blankenship & Yayanos 2005) but with two differences: (i) *Rivacindela* DNA samples were isolated from whole specimens instead of gut contents alone, and (ii) the targeted gene was *cob* instead of *cox*1. Hence, I expanded the study to seven *Rivacindela* specimens (Table 1) which mitochondrial *cob* sequences displayed many double peaks elsewhere (Pons *et al*., unpublished data), presumably due to coamplification of prey sequences. They were collected in five ephemeral lakes from Western Australia (Goongarrie, Weelhamby, Moore, Giles and Goorly; an area between 29°59′38″ and 29°11′29″S, and 116°27′51″ and 121°16′44″E). Preliminary taxonomic determinations done in the field and based on external morphology suggest that specimens 67a and 68b were *Rivacindela blackburni*, individual 68a *Rivacindela aurifodina* and the remaining ones new species (W. D. Sumlin, personal communication).

Since PCR products of these specimens produced unreadable sequences, they were cloned using the pMOS-Blue blunt ended cloning kit (Amersham Pharmacia Biotech). PCR clone amplifications were purified with Gene Clean II (BIO101), sequenced directly for both strands with the BigDye Terminator Cycle Sequencing Kit and an ABI PRISM 3700 DNA Analyser (Applied Biosystems) and edited with SEQUENCHER 3.1. Several clones (3–14, see Table 1) were sequenced for each specimen depending on availability and intraindividual sequence divergence. Sequences from clones of the same individual that included one nucleotide difference relative to another, in few cases two changes, were considered as *Taq* polymerase errors, and hence only their 50% majority rule consensus sequence was considered for further analyses. *Rivacindela* specimens showed from one to seven different sequences (Table 1). Cloned sequences also presented 358 bp and perfect protein coding translation excluding three of them (S15 of 352 bp, S24 of 360 bp and S25 of 353 bp). Protein translation of the latter also revealed many nonsynonymous substitutions and stop codons relative to mitochondrial *cob* sequences of *Rivacindela* specimens. Clustal alignment of these sequences, performed in DAMBE version 4.2.13 using the default parameters, evidenced the presence two deletions of 5 bp and a single base in sequences S15 and S25 (positions 248–252 and 307, respectively). In addition, sequences S24 and S25 had an insertion of a single base in the position 265, and sequence S24 another one in the position 339.

A maximum likelihood phylogenetic tree (Fig. 1) was built with the 30 *cob* sequences obtained from the 14 *Rivacindela* specimens studied here, directly from PCR products or clones, plus 20 previously confirmed mtDNA *cob* sequences from *Rivacindela* individuals that are representative of the within-genus variation (Pons *et al*. unpublished data). Tree showed relationships and divergences within and between sequences of both groups. These sequences were also compared against public databases using the BLAST N algorithm to investigate their taxonomic status (Table 1).

Alignment, phylogenetic and BLAST results indicated that the 30 sequences could be assigned to three groups. (i) Sequences S09 and S18, from specimens 68a.9 and 140.5, were 'true' *Rivacindela* mtDNA sequences since they show low divergence (<6%), high BLAST scores (>e-160), short branches and absence of indels, stop codons and nonsynonymous substitutions relative to the 20 *Rivacindela* mtDNA sequences obtained elsewhere (Pons *et al*. unpublished data). (ii) Seven sequences (S07, S10, S14, S15, S24, S25 and S29) from specimens 140.7, 91.6, 94a.3 (2), 146.3 and 146.7 (2) were pseudogenes because, despite being clustered with 'true' *Rivacindela* mtDNA, they showed indels, stop codons, high frequency of nonsynonymous substitutions and more nucleotide substitutions per

branch. This would explain why they were outside and basal to the cluster including 'true' mtDNA of the same populations (68, 94a, 140 and 146; Fig. 1). (iii) The last group was composed of sequences very divergent (>20%) relative to 'true' *Rivacindela* mtDNA but showed sound protein translation and high identity levels (BLAST *E* values > e-70), suggesting they are orthologous *cob* sequences from other organisms. They were obtained from PCR products or multiple clones per specimens and matched those sequences isolated elsewhere from the insect orders Diptera, Hymenoptera, Isoptera and Coleoptera (except cicindelids, see below; Table 1 and Fig. 1). As these taxa are certainly no parasites of tiger beetles, we conclude that flies, bees, termites and carabid beetle *Blackburnia* are all part of the *Rivancindela* prey diet. The few observations made on the field corroborate that flies compose the main diet of *Rivacindela* but also ants, wolf spiders and other small insects (D. Sumlin, personal communication). Flies were also reported elsewhere as main preys of tiger beetles (Hoback *et al*. 2001). Taxonomic determination of our anonymous sequences up to species level using BLAST could only be achieved for two sequences (S00 and S01) because of full nucleotide identity. The other sequences showed lower identity (around 85% similarity) that was futile for species determination but high enough to identify with assurance order, family and perhaps genus.

Four sequences (S08, S17, S21 and S23) matched sequences from other cicindelids, but due to the lower similarity (85%), we could not discriminate whether they could be congeneric predation or contaminations. This behaviour has been observed in *Rivacindela hudsoni* feeding on *Rivacindela gagei* and *Rivacindela lutamatrix* (D. Sumlin, personal communication) and in other cicindelids (D. W. Brzoska, personal communication; Hoback *et al*. 2001). Unfortunately, clones S19 and S28 were a contamination as their sequences showed 98% identity with sequences of *Cicindela tranquebarica* and *Cicindela cazieri*, which are common group of study in our laboratory and only found in North America. However, this contamination can be considered negligible (just one clone out of the eight screened in specimen 140.5, and one out of 11 in individual 146.7) and is probably associated with the difficulty of amplifying degraded prey DNA, and hence magnifying external contaminants.

There are several evidences strongly suggesting that most of the *cob* sequences amplified from genomic DNA extracted from endemic Australian *Rivacindela* specimens are from prey remains rather than contamination: (i) field observations corroborated a posteriori that flies are the main preys; (ii) bee sequence detected in two *Rivacindela* specimens were identical to Australian bee populations in a marker that is highly variable and generally diagnostic at biogeographic level; (iii) they matched genera (see Table 1) distributed or endemic of Australasia; (iv) evidences of alien sequences were centred around most specimens of few populations and not randomly among populations and individuals as should be expected if it was contamination, and (v) our laboratory does not work neither with flies nor bees, and controls were negative.

Results shown here corroborate that DNA-based approach using universal primers is a great tool to study prey spectrum, and that genomic DNA extracted from whole predatory specimens is a suitable sample. This approach is ideal as preliminary study and after that perform easier and more accurate analyses, e.g. designing species-specific primers for each prey (Jarman *et al*. 2004) or removing predator copies (Blankenship & Yayanos 2005). Finally, (i) results suggest that *cob* sequence seems to work as well as *cox*1 gene to determine species status, and hence they could also be used as DNA barcode; and (ii) the rapid increase of databases will make BLAST algorithms an useful, fast, and accurate tool for DNA-based species determination.

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