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The first DNA barcodes for the Australian platypus tick *Ixodes ornithorhynchi* Lucas, 1846 (Acari: Ixodidae) to facilitate conservation efforts for a declining parasite and its host

Mackenzie L. Kwak^a, Josh Griffiths^b, Dylan Barry^c, Mikayla Begent^c, Tyrone Hoang^c, Luisa Taafua^c, Anthony Chiovitti^c

^a Evolutionary Biology Laboratory, Department of Biological Science, National University of Singapore, 16 Science Drive 4, 117558, Singapore.

^b CESAR, 293 Royal Parade, Parkville Victoria 3052 Australia.

^c Gene Technology Access Centre (GTAC), 77 Story Street, Parkville, Victoria 3052 Australia.

ABSTRACT

Ixodes ornithorhynchi is one of Australia's most cryptic tick species and is found only on the platypus (*Ornithorhynchus anatinus*). The first cytochrome c oxidase subunit 1 (CO1) sequences for this species are presented to facilitate molecular identification and conservation of both this tick species and its host.

Keywords COI, parasite conservation, platypus inbreeding, bottleneck

Zoobank <http://zoobank.org/65AEFA11-8A3F-425B-B016-A7FBB8BBCF9>

Introduction

Ixodes ornithorhynchi is a host-specific tick of the Australian platypus (*Ornithorhynchus anatinus*) (Roberts, 1970). In recent decades, many platypus populations have become fragmented and both the host and parasite are believed to have gone extinct on the mainland in at least one Australian state (Grant and Temple-Smith, 2003). The platypus has an extensive range spanning most of eastern Australia, but both it and *I. ornithorhynchi* are of conservation interest due to this pattern of decline (Menkhorst and Knight, 2011). Although tick population sizes can be monitored using morphological identification, assessing the conservation status of a tick population often requires molecular study to identify levels of genetic diversity and instances where genetic bottlenecks form. To better facilitate research on the conservation biology of *I. ornithorhynchi* and the platypus in Australia, the first DNA barcodes of *I. ornithorhynchi* are presented.

Material and methods

Twelve tick specimens (representing 9 nymphs and 3 adult females) collected from two platypodes (*O. anatinus*) in Jackson Creek, Sunbury, Victoria, Australia were identified using morphology-based keys provided by Roberts (1970) under a light microscope. Scanning electron micrographs were taken of specimens from which DNA barcodes were generated. These were taken using a Hitachi TM3030 electron microscope and are presented in Figure 1.

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Corresponding author
Mackenzie L. Kwak:
mackenziekwak@gmail.com

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Figure 1 Ventral view of *Ixodes ornithorhynchi* specimens from which DNA barcodes were generated. A – Adult female; B – Nymph.

Total genomic DNA was extracted from 12 individuals using the Nucleospin Tissue Kit (Machery-Nagel, Germany) following the manufacturer's protocols. The mitochondrial COI gene was targeted with a primer combination adapted from Kwak *et al.* (2017). This comprised a forward primer, HCO2064 (5'-GGT GGG CTC ATA CAA TAA ATC C-3') and a reverse primer, HCO1240 (5'-CCA CAA ATC ATA AAG ACA TTG G-3') yielding a fragment of 846 bp in length. The COI gene segment was amplified from the purified genomic DNA using the Phusion HotStart Taq DNA polymerase (New England Biolabs, USA) with accompanying reagents according to the manufacturer's protocols. The PCR was run on a BioRad model T100 thermocycler with a temperature cycle encompassing denaturation at 98 °C for 30 s, annealing at 48 °C for 30 s, and primer extension at 72 °C for 45 s. The cycle was repeated 30 times and bracketed by an initial denaturation step at 98 °C for 2 min and a terminal extension step at 72 °C for 5 min. PCR products were visualised by gel electrophoresis (1%), stained using SYBRSafe (Invitrogen, USA) and compared against size standards (Quick Load PCR Marker #N0475S, New England Biolabs, USA). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) following the manufacturer's protocol and were submitted to the Australian Genome Research Facility (AGRF) for bidirectional Sanger sequencing using the same primers as those used for PCR.

A phylogenetic tree was constructed using the program MEGA6 (Tamura *et al.*, 2013). Sequences were trimmed then aligned using the ClustalW function. The phylogeny was inferred using the neighbour-joining method with 10,000 bootstrap replications. Sequences of *I. ornithorhynchi* were compared against those of other *Ixodes* species present on GenBank and are presented in Table 1. Species used for comparison include those from the main Australian subgenera as well as the *Afrixodes* and *Partipalpiger* which exhibit morphological similarities with *I. ornithorhynchi*, as listed by Clifford *et al.* (1973). The southern reptile

Table 1 *Ixodes* COI sequences used for the molecular analysis.

Subgenus	Species	Genbank accession number
<i>Ceratixodes</i>	<i>Ixodes uriae</i>	KX360345.1
' <i>Coxixodes</i> '	<i>Ixodes ornithorhynchi</i>	MH666077
	<i>Ixodes ornithorhynchi</i>	MH666078
<i>Endopalpiger</i>	<i>Ixodes fecialis</i>	KX673872.1
	<i>Ixodes fecialis</i>	KX673871.1
<i>Exopalpiger</i>	<i>Ixodes australiensis</i>	KX673865.1
	<i>Ixodes australiensis</i>	KX673864.1
	<i>Ixodes australiensis</i>	KX673863.1
	<i>Ixodes tasmani</i>	KY213772.1
	<i>Ixodes tasmani</i>	KX673869.1
	<i>Ixodes victoriensis</i>	KX673874.1
	<i>Ixodes victoriensis</i>	KX673873.1
	<i>Ixodes woyliei</i>	KX673881.1
	<i>Ixodes woyliei</i>	KX673880.1
	<i>Ixodes woyliei</i>	KX673879.1
<i>Sternalixodes</i>	<i>Ixodes cornuatus</i>	KY213793.1
	<i>Ixodes cornuatus</i>	KY213792.1
	<i>Ixodes cornuatus</i>	KY213791.1
	<i>Ixodes hirsti</i>	KY213775.1
	<i>Ixodes hirsti</i>	KY213774.1
	<i>Ixodes hirsti</i>	KY213773.1
	<i>Ixodes holocyclus</i>	KY213783.1
	<i>Ixodes holocyclus</i>	KY213782.1
	<i>Ixodes holocyclus</i>	KY213781.1
	<i>Ixodes trichosuri</i>	KY213777.1
	<i>Ixodes trichosuri</i>	KY213778.1

tick (*Bothriocroton hydrosauri*) was used as an outgroup (GenBank reference: FJ584427.1). Bootstrap values <45 are not shown as the purpose of the phylogeny is to demonstrate the distinctiveness of the species from its relatives rather than to infer, and support, deeper evolutionary relationships.

Results

The adult female specimens were identified as *I. ornithorhynchi* based on a combination of morphological features including unarmed coxae, the absence of cornua and auriculae, large porose areas, hypostomal dentition of 3/3, long palps in which all four articles are distinct and without spurs, and an anal groove which does not meet posteriorly. Nymphs were identified as *I. ornithorhynchi* based on a combination of morphological features including unarmed coxae, the absence of cornua and auriculae, hypostomal dentition of 2/2, long palps in which all four articles are distinct and without spurs, and an anal groove which does not meet posteriorly.

Unique sequences were obtained at the COI locus for two *I. ornithorhynchi* specimens representing one nymph and one adult female tick (Table 1). All other sequences were identical to that of the nymph.

Figure 2 Phylogenetic relationships of *Ixodes ornithorhynchi* at the COI locus with published reference sequences of other *Ixodes* species. Evolutionary history was inferred using the neighbour-joining method supported with a bootstrap test of 10,000 replicates (bootstrap values >45 shown). The reptile tick *Bothriocroton hydrosauri* was used as an outgroup.

Phylogenetic analysis (Figure 2) placed *I. ornithorhynchi* in a distinctly separate clade from all other tick species to which it was compared. Although several morphological similarities exist between *I. ornithorhynchi* and members of other subgenera, *I. ornithorhynchi* does not fall within any of these clades.

Discussion

Based on the phylogenetic tree presented in Figure 2, *I. ornithorhynchi* appears to be distinct from all other species to which it was compared. This is unsurprising, as the higher classification of *I. ornithorhynchi* has been far from settled historically due to the morphological distinctiveness of the species. Since Schulze (1941) erected the monotypic genus *Coxixodes* for *I. ornithorhynchi* in a short passage of German, few taxonomists have accepted and used it, even though it was reclassified as a subgenus. Roberts (1970) completely ignored the subgenus and Clifford *et al.* (1973) avoided the use of *Coxixodes*, citing limited available data to support its validity.

The evolutionary relationship between *I. ornithorhynchi* and the other *Ixodes* subgenera remains poorly understood. The genus *Ixodes* comprises over 240 known species, many of which are still without molecular data (Guglielmone *et al.*, 2010). This lack of data renders a total comparison of species at the COI locus impossible. As more DNA sequences of the COI locus become available for members of *Ixodes*, it may become possible to determine the evolutionary relationships between closely related *Ixodes* species. However, as demonstrated by Kwak *et al.* (2017), the COI marker only appears useful in distinguishing the evolutionary relationships between closely related members of the *Ixodes* genus. Therefore, the phylogeny presented in Figure 2 is only valuable to demonstrate the distinctiveness between *I. ornithorhynchi* and the other *Ixodes* to which it was compared. Analysis of more

highly conserved genetic markers will be required to make inferences about the evolutionary relationships amongst species of this highly diverse genus.

Although DNA barcodes of *I. ornithorhynchi* are useful for delimiting species boundaries, they may also serve a purpose in future attempts to monitor and map the population dynamics of platypus ticks and their hosts, both past and present. Nieberding and Olivieri (2007) suggested that parasites could be used to map past population events in their hosts such as rapid range expansions or contraction events causing genetic bottlenecks. Biek *et al.* (2006) successfully demonstrated this when they showed how an examination of a directly transmitted parasite could be used to map the recent demographic history of its host population. As *I. ornithorhynchi* is a directly transmitted, host specific parasite, and the only tick species regularly found on the platypus, it is a good candidate for such study. Haplotype diversity at the COI locus could be used as a proxy to estimate historical levels of gene flow between platypus populations. Although COI is a maternally inherited marker, it may still prove useful for providing a raw estimation of population stability. Previously, COI has been used in New Zealand in endangered *Amblyomma sphendonti* populations to estimate gene flow and intrapopulation genetic diversity (Miller *et al.*, 2007). Measuring haplotype diversity at the COI locus could similarly be a valuable way to infer and monitor gene flow and relative genetic diversity in *I. ornithorhynchi* populations to ensure the continued survival of one of Australia's most cryptic arthropods across its range.

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