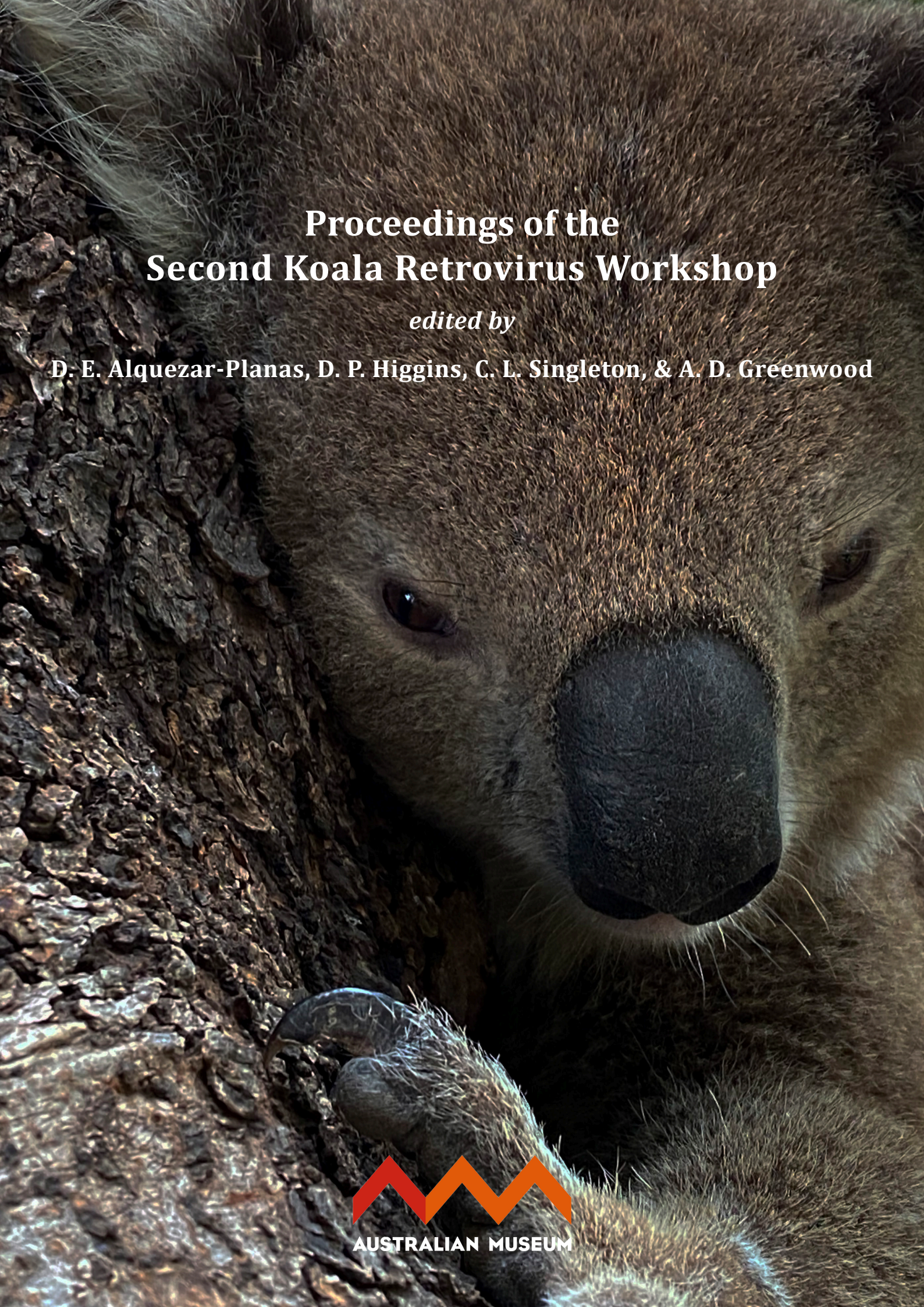
# Proceedings of the Second Koala Retrovirus Workshop

*edited by*

D. E. Alquezar-Planas, D. P. Higgins, C. L. Singleton, & A. D. Greenwood



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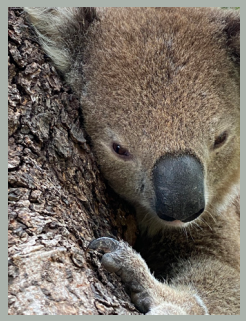

Cover photo
by Damien P. Higgins

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# The Koala Retrovirus: Lessons Learned from the Koala Genome

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Abstract. The establishment of the Koala Genome Consortium in 2013 culminated in the publication of the first fully assembled koala genome. An international initiative involving 29 institutes across the globe, the publication has led to a much greater understanding of koala biology including knowledge on gene families putatively associated with detoxification of eucalypt leaves and the species' ability to taste and smell plant secondary metabolites. Similarly, the genomic resource has enabled comparative assessments facilitating immunogenomics, population genomic analysis, and, for the first time, genome-wide assessments of the koala retrovirus (KoRV). This summary outlines how the koala genome has increased our capacity to understand the genetics of KoRV—from a deeper understanding of KoRV viral subtypes and their recombinants to preferences for viral integration across the host genome.

## Introduction

The koala (*Phascolarctos cinereus*) is an arboreal marsupial species that is endemic to the eastern Australian mainland and is the only living representative of the family Phascolarctidae. Having a unique biology, koalas are characterized by their evolutionarily unique physiological adaptations, such as their capacity to thrive almost exclusively on the consumption of eucalyptus leaves (Moore & Foley, 2000). In recent years, koala populations have experienced significant declines, which have been attributed to a range of factors including widespread habitat loss through land clearing and extreme climactic conditions such as those preceding and associated with the 2019–2020 summer bushfires (Phillips *et al*., 2021). Susceptibility to various infectious diseases such as chlamydiosis and potential pathogens such as the koala retrovirus (KoRV), has created additional selective pressures that collectively have impacted most koala populations to some degree. The multifactorial nature of these declines has underpinned the complexities of managing the species, particularly as populations across the range are threatened through a combination of these different factors.

Considering these widespread declines, the Koala Genome Consortium was established with the purpose of generating the first high-quality koala genome assembly to be used as a resource by researchers to enact measurable conservation outcomes (Johnson *et al*., 2014). The culmination of this work offers multiple insights into the species (Johnson *et al*., 2018), but additionally provides a unique resource for comparative genomic applications, including the study of KoRV, found across the koala genome.

KoRV is a gammaretrovirus that is in the process of endogenization across the koala genome. Endogenous retroviruses (ERVs) descend from exogenous retroviruses that infected a host germline and have since propagated through vertical transmission via parent to offspring. While most ERVs colonized their host genomes millions of years ago, KoRV is estimated to have entered the koala germline much more recently (Ishida *et al*., 2015) and may spread through either vertical or horizontal transmission. Belonging to the Retroviridae family of viruses, KoRV replication commences with the conversion of retroviral RNA via reverse transcription into double stranded DNA within the host cell. The viral DNA subsequently becomes integrated

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into the host genomic DNA and inevitably forms a permanent alteration that may be studied through the koala genome.

## Advancing our knowledge of KoRV through use of the koala genome or koala genome resources

A mere 10 years ago, KoRV sequence diversity was assumed to be comprised of a single genetic subtype, endogenous KoRV-A (Quigley & Timms, 2020). However, the years that followed outlined a much more complex evolutionary picture of KoRV diversity, including the identification of various other subtypes, such as exogenous KoRV-B, which utilizes a different receptor binding domain (THTR1) than does KoRV-A (Pit1) (Xu *et al*., 2013). Wider adoption of high-throughput sequencing applications has also aided in our understanding of KoRV sequence diversity. While much of the KoRV provirus has remained remarkably conserved, most sequence diversity has been characterized across the *env* hypervariable region within the receptor binding domain used for mediating cellular infection (Chappell *et al*., 2017; Sarker *et al*., 2021).

Despite these advances, the lack of a koala reference genome has complicated the ability to pair positional information within the host with KoRV sequence diversity. KoRV analysis is further compounded by the limited diversity across viral genes and the repetitive Long Terminal Repeat sequences that are characteristic of retroviral elements, which make sequence assembly using short-read applications methodologically challenging. Thus, while KoRV diversity could be characterized, genetic insights into KoRV have been done so in aggregate, where KoRV reads (potentially originating from various KoRV proviruses across the genome) are mapped to a full length assembled provirus (Löber *et al*., 2018). In this manner, assembling specific KoRV-like proviruses, pinpointing the genomic location of these integrants, and studying the effects that these integrations may have conferred to the host, was not possible.

In the past five years, through the analysis and utility of the koala genome, several studies have expanded our knowledge of KoRV that would otherwise not have been possible without access to this resource. Notably, a study by Hobbs *et al*., outlined the first comprehensive picture of full length endogenous KoRV proviruses within a single koala, achieved through the analysis of long PacBio sequence reads later used to assemble the first koala genome (Johnson *et al*., 2018). Analysis of the sequencing reads provided several additional insights including positional data on integration sites across the genome; the characterization of a newly identified endogenous recombinant retroelement termed recKoRV—the result of a recombination of an older ERV termed *Phascolarctos* endogenous element (PhER) and KoRV; and putative evidence of somatic cell integration by exogenous KoRV (Hobbs *et al*., 2017).

A key area of KoRV research and retrovirology that has flourished with access to koala genome resources is the study of viral integration sites. As a young retrovirus, integration site analysis of KoRV provides a unique opportunity to study retroviral endogenization within a mammalian host in real-time. As a North-South cline to viral infection appears the most likely explanation for KoRV infection and expansion across the koala genome; the resource has provided opportunities to study KoRV integration patterns across time through the analysis of historical and contemporary museum specimens (Cui *et al*., 2016).

Previous studies have shown that retroviral genera display differing integration site preferences (Kvaratskhelia *et al*., 2014). However, while integration into a specific genomic locus is random, retroviruses within the same family are statistically more likely to integrate within specific host genome features (Lafave *et al*., 2014). The recent development of a novel genetic assay termed sonication inverse PCR (SIP) has aided integration site analysis, particularly when coupled with long-read PacBio sequencing and comparative assessment to the koala genome (Alquezar-Planas *et al*., 2021). The tool was successfully applied to comprehensively compare KoRV and recKoRV integration sites of an unrelated koala to the reference genome. In doing so, the role that older ERVs play in the disruption and remobilization of active retroviruses like KoRV at the earliest stages of endogenization within the koala genome was able to be examined (Löber *et al*., 2018).

Another application of viral integration site analysis made possible through comparative assessment to the koala reference genome is the study of pathogenesis. Insertional mutagenesis mediated through viral integration is one of several known mechanisms by which a retrovirus may cause cancer in its host. These integrations may result in several deleterious effects, including the disruption of oncogenes and the up or down regulation of gene expression (Bushman, 2020). Like several other gammaretroviruses with known oncogenic capacity, KoRV has been long suspected of increasing cancer prevalence in koalas, particularly as lymphomas and leukaemias occur in high prevalence across the species. Through the analysis of paired healthy and neoplastic tissue from 10 koalas, a recent study by McEwen *et al*. (2021) provided the first supportive evidence of KoRV to underlie elevated cancer rates in koalas. The analysis of the paired tissue provided support for the identification of up to 172 integration sites uniquely found within the neoplastic tissue but absent in healthy tissue. Through the analysis, evidence for KoRV involvement in cancer development via different viral mechanisms are proposed (McEwen *et al*., 2021).

## Conclusions and future perspectives

Through the analysis of koala genome reads, or the comparative assessments of proviral KoRV mapped back to the koala genome, the last five years has uncovered insights into KoRV biology, KoRV subtype diversity and the effects of viral integration on putative disease manifestations. With the decreased cost of sequencing technologies, the complete sequencing and annotation of hundreds of koala genomes across the species range is not far away. In fact, a large whole genome sequencing project lead by the University of Sydney with funding support from the NSW and the Australian Federal Government is presently underway and seeks to achieve this. Over the coming months, 450 koala genomes sequenced from across the species range will be uploaded into the public domain to support vital genomics research. The resource will enable key questions on koala biology, health and disease, and adaptations to climate change (among others) to be explored. The comparative assessment of this data is likely to provide further insights into KoRV that presently remain poorly understood. Retroviral infection processes, used by KoRV to propagate and spread, are one such area. Evidently, while some KoRVs are transcriptionally active, others are defective (as characterized by the disruption of open reading frames) but may still remobilise through

various mechanisms such as retrotransposition. Also, a virus in the process of endogenization is likely to propagate and spread using a broad range of mechanisms that differ to one that is exclusively exogenous or has seemingly reached equilibrium within its host. The complex evolutionary processes undergone by KoRV since its emergence and spread throughout the koala host is likely only to be fully uncovered through its analysis across multiple genomes, especially in related animals but also throughout its range. Another area of knowledge likely to continue to grow is our understanding of KoRV sequence diversity across the species. Recent technological developments have already enabled a much deeper understanding of viral subtypes that can be pinpointed to specific locations across the genome. Access to these same resources is likely to expand on how KoRV integrations may contribute to other diseases via immune modulation, either individually or in conjunction with other infectious agents. The years that follow are likely to provide fertile grounds for uncovering KoRV mysteries that would not otherwise be possible without access to the first koala genome.

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