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The evolution of bitter taste receptors in the eucalypt-specialist koala

(ユーカリスペシャリストであるコアラの苦味受容体の進化)

General Introduction

The koala (*Phascolarctos cinereus*) is a unique and highly specialized arboreal folivore, the sole extant member of the family Phascolarctidae. Its dietary niche is remarkably narrow, centered almost exclusively on the foliage of the genus *Eucalyptus*. Eucalypt leaves represent an exceptionally challenging food resource, characterized by low nutritional value, high fiber content, and an array of potentially toxic plant secondary metabolites (PSMs), including volatile terpenes and non-volatile compounds such as tannins and formylated phloroglucinol compounds (FPCs). To survive on such a diet, koalas have evolved complex physiological adaptations, including specialized gut microbiota and an efficient hepatic detoxification system.

Despite being classified as dietary specialists, koalas exhibit significant variation in food preferences. Wild observations reveal striking individual and regional differences in eucalypt selection. Within the same forest, individual koalas often display a fussy feeding behavior, persistently selecting specific trees while rejecting others of the same species. Furthermore, regional variations are evident across the Australian continent; for example, southern populations frequently consume eucalypt species containing chemicals like unsubstituted B-ring flavanones (UBFs) that are largely avoided by northern populations. These patterns suggest that koalas have evolved sophisticated mechanisms to evaluate the chemical composition of leaves before ingestion, allowing them to navigate the phytochemical mosaic of their vast habitat. The preference differences among individuals and regions indicate that the koala's sensory system is not a fixed species-wide trait but may be tuned

to local chemical environments.

The sense of taste serves as the primary gatekeeper in this pre-ingestive evaluation process. Among the five basic tastes, bitter taste perception is a critical innate defense mechanism for detecting and avoiding the ingestion of harmful substances. This perception is mediated by the Taste 2 Receptor (TAS2R) family, a group of G-protein coupled receptors expressed on the tongue. In many mammals, the number and diversity of TAS2R genes reflect their dietary breadth and environmental chemical complexity. Recent genomic investigations have revealed that the koala possesses a significantly expanded repertoire of approximately 28 functional TAS2R genes compared to other Australian marsupials. This expansion is thought to provide the sensory sensitivity required to manage the high toxin load of a eucalyptus diet.

However, the functional and evolutionary significance of this genetic expansion remained largely unexplored. It was unclear how these receptors contribute to the observed individual and regional differences in dietary habits of koalas. The diversification of TAS2R genes may facilitate local adaptation by fine-tuning bitter sensitivity to the specific PSM profiles of local eucalypt species. This thesis addresses these questions by integrating large-scale population genomics and detailed functional characterization. By analyzing TAS2R genetic variation across the koala's geographic range and characterizing their functional responses to eucalypt-derived compounds, this study aims to elucidate the molecular basis of sensory adaptation. Understanding these mechanisms is not only vital for mammalian evolutionary biology but also holds practical importance for koala conservation, particularly in ensuring dietary compatibility for translocation programs in a changing environment. By bridging the gap between genomic diversity and ecological function, this research provides a new framework for understanding how specialized herbivores survive in toxic landscapes.

Chapter 2: Adaptation to eucalypt diversity through bitter taste receptor diversification

In Chapter 2, I investigated the genetic diversity and evolutionary drivers of TAS2R genes in the koala across its broad geographic range. I analyzed whole-genome sequencing data from 396 wild koalas categorized into five major genetic clusters: Northern Queensland, Southeast Queensland and Northern New South Wales, Mid-Coast

New South Wales, Southern New South Wales, and Victoria. The primary objective was to characterize TAS2R variation at the population level and determine how local eucalypt diversity influences the evolution of these sensory genes.

A significant finding was the variation in the number of functional TAS2R genes among individuals, which ranged from 24 to 28. This copy number variation was ecogeographically structured; for instance, the Victorian population exhibited the highest mean number of intact genes, while the Southern New South Wales population had the lowest. The presence of segregating pseudogenes—genes that are functional in some individuals but pseudogenized in others—suggests that the koala's bitter taste repertoire is dynamic and potentially responding to regional dietary challenges. This variation may indicate that the sensory capacity of the species is not uniform but varies according to local environmental requirements.

Across the 28 identified TAS2R genes, I found substantial allelic diversity, identifying 255 nucleotide and 148 amino acid haplotypes. While nucleotide diversity generally followed a north-to-south decline consistent with neutral genomic trends, amino acid diversity patterns suggested that specific receptors are being maintained or diversified in response to local selective pressures. Population structure analysis using PCA and fixation index (F_{st}) confirmed that TAS2R genes exhibit strong genetic differentiation. Notably, the differentiation in southern populations exceeded what would be expected from geographic isolation alone, suggesting that functional divergence in bitter taste perception is a key feature of koala evolution.

To identify the ecological drivers of this differentiation, I employed genotype-environment association and partial redundancy analysis. I revealed that dietary resource availability, specifically the species richness of eucalyptus subgenera, is the primary driver of TAS2R evolution. Notably, the richness of the subgenus *Eucalyptus* (formerly *Monocalyptus*) exerted a selective force nearly twice as strong as that of the preferred subgenera *Alveolata* and *Symphyomyrtus*. Since *Monocalyptus* species are rich in deterrent UBFs, this finding provides robust evidence that the need to detect and avoid specific phytochemicals has shaped the sensory landscape of the koala. The study successfully linked genetic variation to the specific phytochemical mosaic of the Australian environment.

Furthermore, I analyzed signatures of natural selection using Tajima's D statistics. Many TAS2R genes exhibited significantly higher values than genome-wide background, pointing to positive selection and local adaptation. Positive Tajima's D values for several receptors indicated the action of balancing selection, which likely maintains a diverse set of functional alleles within populations. This strategy allows koalas to handle the complex and varying chemical defenses of eucalypt forests. The high level of amino acid diversity maintained in certain populations further supports the hypothesis that these receptors are under constant evolutionary pressure to adapt to local dietary toxins.

In summary, Chapter 2 demonstrated that the diversification of koala TAS2Rs is a result of multidimensional adaptation to local vegetation. The strong correlation between receptor variation and the presence of chemically defended eucalypt species indicates that the koala's bitter taste system is finely tuned to its local dietary environment. These findings provide the evolutionary basis for the observed regional differences in food preference and establish that sensory adaptation is a fundamental component of the koala's success as a dietary specialist.

Chapter 3: Functional evolution of bitter taste receptors

In Chapter 3, I focused on bridging the gap between the genetic variation identified in the previous chapter and the actual physiological responses of koala TAS2R. I employed a multidisciplinary approach, combining cell-based functional assays, evolutionary network analysis, and structural modeling to elucidate how the expanded repertoire of TAS2R facilitates the koala's survival on a diet dominated by chemically defended eucalypt foliage.

The methodology centered on a heterologous expression system where 28 intact koala TAS2R genes were transfected into HEK293T cells. These cells were co-transfected with a chimeric G-protein and a calcium-binding photoprotein to measure receptor activation through luminescence assays. This comprehensive screening involved a panel of 44 bitter compounds, including known vertebrates agonists and PSMs specifically isolated from eucalypt leaves, such as terpenes, flavonoids, and glycosides. This effort represents the first

systematic functional mapping of the entire TAS2R repertoire in the koala, moving beyond the single previously deorphanized receptor.

I successfully deorphanized eight of them, demonstrating the ability to detect diverse eucalypt-derived plant secondary metabolites, such as terpenes, flavonoids, and glycosides. TAS2R710A exhibited the broadest tuning by responding to seven agonists including caffeine, coumarin, and resveratrol. TAS2R4 followed closely, detecting six agonists, primarily polyphenols. In contrast, other receptors had relatively narrow breadth. For instance, TAS2R705A and TAS2R705C were narrowly tuned to single glycosides—arbutin and salicin, respectively. Given that these receptors are orthologs of the primate β -glucoside receptor TAS2R16, their functional specificity appears highly conserved across mammalian evolution, serving to detect cyanogenic glycosides found in specific eucalypt species.

A pivotal discovery in this chapter was the functional divergence observed between the two major haplotypes of TAS2R728A. Genetic analysis in Chapter 2 had noted significant regional differences in the frequency of these haplotypes. Dose-response assays revealed that Haplotype 1, which is prevalent in northern koala populations, is significantly more sensitive to pinocembrin and absinthin than Haplotype 2, which dominates southern populations. Pinocembrin is an UBF known to act as a potent feeding deterrent. To pinpoint the molecular basis of this sensitivity shift, I conducted site-directed mutagenesis, creating various hybrid receptors. This revealed that the simultaneous substitution of two specific amino acid residues—arginine to serine at position 78 (R78S) and valine to isoleucine at position 85 (V85I)—is necessary and sufficient to reduce receptor sensitivity.

Evolutionary analysis further enriched these findings. Rooting a median-joining network with the common wombat as an outgroup suggested that the low-sensitivity haplotype (Hap2) represents the ancestral state in the koala lineage. This implies that the high-sensitivity haplotype (Hap1) is a derived trait that likely evolved as northern koalas faced different selective pressures or developed a more selective foraging strategy to avoid toxic UBFs.

To understand the structural mechanism, I utilized AlphaFold2 for 3D modeling and performed

molecular docking simulations. Mapping the mutations onto the Class T GPCR numbering system positioned residue 78 at the extracellular boundary of transmembrane helix 3 and residue 85 within its upper segment. Interestingly, docking simulations indicated that neither residue directly interacts with the ligands. Instead, I proposed that these residues act as structural modulators. The bulky, positively charged arginine in Hap1 likely facilitates ligand access or stabilizes an active conformation in the receptor vestibule, whereas the smaller serine in the ancestral Hap2 reduces entry efficiency.

In conclusion, Chapter 3 established that koala bitter taste sensitivity is not uniform but is finely tuned at the molecular level to the local phytochemical environment. The functional divergence of TAS2R728A provides a clear mechanistic link to the regional dietary differences observed in wild populations. Specifically, the lower sensitivity of southern koalas likely represents a strategy of sensory desensitization, allowing them to exploit the abundant, UBF-rich *Monocalyptus* foliage that would be rejected by northern individuals. This sensory tuning proves to be a fundamental driver of ecological adaptation in this iconic specialist herbivore.

General Discussion

This doctoral research aimed to elucidate how the diversification and functional evolution of TAS2R drive local adaptation to chemically diverse eucalypt environments in the koala. By integrating population genomics with cell-based functional assays, the study provides a comprehensive view of the molecular mechanisms underlying dietary specialization and intraspecific variation in food preference.

The genomic analysis revealed that TAS2R genetic differentiation among koala populations is not merely a byproduct of geographic isolation but is significantly driven by local environmental factors, specifically dietary resource richness. I found that the richness of the eucalyptus subgenus *Eucalyptus* (formerly *Monocalyptus*) is a primary driver of TAS2R diversification. This suggests that the evolutionary pressure to navigate PSMs has led to regional specializations in the koala's sensory repertoire, where genetic–ecological links are robustly supported by multivariate association analyses.

Functional assays deorphanized eight koala TAS2Rs, identifying ligands such as terpenes, flavonoids,

and glycosides. A pivotal finding was the functional divergence of TAS2R728A. Haplotype 1, prevalent in northern populations, exhibited significantly higher sensitivity to pinocembrin—a deterrent UBFs—compared to Haplotype 2, which dominates southern populations. Site-directed mutagenesis confirmed that simultaneous substitutions at positions 78 and 85 (R78S and V85I) are required to reduce this sensitivity. This suggests a sensory desensitization strategy in southern koalas, allowing them to exploit UBF-rich *Monocalyptus* species that are typically avoided by northern individuals.

The evolutionary analysis identified the low-sensitivity haplotype (Hap2) as the ancestral state. A proposed evolutionary scenario suggests that while the ancestral koala population possessed the low-sensitivity haplotype, the high-sensitivity Hap1 emerged later in the north. The maintenance and dominance of the ancestral Hap2 in the south provided a selective advantage, facilitating niche expansion into forests where chemically defended foliage is abundant. This highlights how sensory tuning at the molecular level facilitates macroscopic ecological shifts.

Furthermore, the discussion addresses the behavioral diversity observed among southern koalas. Despite the dominance of low-sensitivity receptors, preferences for specific *Monocalyptus* species vary. This discrepancy may be attributed to fine-scale genetic variation, chemical heterogeneity within plants, or physiological factors beyond taste, such as hepatic detoxification via cytochrome P450 enzymes and the role of the gut microbiome in breaking down toxins. I emphasize that taste sensitivity alone does not dictate dietary choices but acts in concert with other physiological constraints.

Broadly, these findings align with ecological theories regarding body size and foraging strategies. Consistent with Bergmann's rule, larger southern koalas likely rely on bulk consumption of foliage, facilitated by reduced bitter sensitivity, whereas smaller northern koalas must forage more selectively for high-quality leaves to meet metabolic demands and avoid toxicity.

In conclusion, this research demonstrated that intraspecific diversity in bitter taste sensitivity may serve as a mechanism for dietary selection and local adaptation. These insights have significant implications for koala conservation, particularly in improving translocation success by matching an individual's sensory profile with the

chemical landscape of potential habitats. Future research should adopt an integrative approach, synthesizing vegetation data, PSM profiles, and genetic variation to ensure the survival of this iconic specialist in a changing environment.