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MITOGENOMIC EVIDENCE REVEALS THE PHYLOGEOGRAPHY OF SULAWESI *Actenoides* KINGFISHER LINKED TO GEOLOGICAL BARRIERS

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ABSTRACT

The genus *Actenoides* (Alcedinidae) comprises brightly colored kingfishers endemic to the Indo-Australian Archipelago. It provides a good model for exploring how geological and ecological factors have shaped diversification across Wallacea. Sulawesi has the highest diversity of *Actenoides* compared to single-island representatives elsewhere. We aimed to study the phylogenomics of *Actenoides* across Indonesia, particularly in Sulawesi, to reveal evolutionary divergence associated with their endemicity using the mitogenome. Phylogenomic relationships were reconstructed using Maximum Likelihood in IQ-TREE. Phylogenomic relationships inferred from Maximum Likelihood in the IQ tree showed divergence between Sulawesi *Actenoides* and *A. concretus*, and between *A. princeps* and *A. monachus*, both supported by high bootstrap values. The genetic p-distance among those species inferred from the NJ tree showed genetic distances of around 8% between the Sumatra (*A. concretus*) and Sulawesi lineages, and 6% between *A. monachus* and *A. princeps*. Furthermore, the population from northeastern Sulawesi for both *A. princeps* and *A. monachus* showed early divergence from other populations; hence, it has deep and distinct lineages even from its closest sister populations in northwestern Sulawesi. These findings highlight phylogenomic differentiation within *Actenoides* and emphasize the role of Sulawesi's complex geological history in promoting inland diversification of Wallacean birds.

Key words: *Actenoides*, endemism, phylogenetics, Sulawesi

INTRODUCTION

Sulawesi, the largest island in the Wallacea region, one of the world's biodiversity hotspots, is characterized by high species richness and endemicity (Myers et al., 2000). The unique biodiversity in Sulawesi is associated with complex geological origins, and it has created the patterns of Area of Endemism (AOE) composited by some well-documented taxa such as Sulawesi Macaca, toad, shrew, and flying lizard (Evans et al., 2003; Evans, Supriatna et al., 2003; Eldridge et al., 2018). Biodiversity in Sulawesi resulted from the colonization of Sunda and Sahul fauna, which, in turn, led to in situ evolution potentially driven by an isolated montane area that began forming around 5 Mya (Stelbrink et al., 2012; Dalsgaard et al., 2014; Nugraha & Hall, 2018).

Behind the rich biodiversity and high endemism in Sulawesi, several pathways of population divergence have been documented through comparative studies across various taxa. Dispersal and vicariance speciations are among the most commonly recognized modes of speciation, while the third process, hybrid speciation, may have been prevalent in forming a hybrid population independent from their parental population (Stelbrink et al., 2012; Mokodongan & Yamahira, 2015; Mandagi et al., 2021; McGuire et al., 2023). Geographical features such as montanes, deep sea, and river systems may act as barriers that facilitate isolation or accommodate connectivity, allowing gene flows to be the main factors for those speciation pathways (Rahbek et al., 2019; Tobias et al., 2020). Therefore, the pattern of avian endemism in Sulawesi may have resulted from a complex interaction between speciation pathways and geographical features that shaped the current Sulawesi avian biogeography. However, while the pattern of Sulawesi avian biogeography is relatively well known based on taxonomic and distributional information, the insitu diversification of birds, which could explain its relationship with Sulawesi's geographical features, is still understudied.

The kingfisher genus *Actenoides* comprises six species: *A. bougainvillei, A. lindsayi, A. hombroni, A. princeps, A. monachus, and A. concretus,* distributed across Southeast Asia and the Pacific, particularly in Sumatra, Borneo, the Philippines, Sulawesi, Bougainville, and Guadalcanal. Sulawesi has the most species and intraspecific populations on a single island, with a total of five subspecies from two species: *A. monachus*, which consists of *A. m. monachus* distributed in north and central Sulawesi and *A. m. capucinus* distributed in east, southeast, and south Sulawesi; *A. princeps* consists of *A. p. princeps* distributed in northeast Sulawesi, *A. p. erythrorhamphus* distributed in northwest, central, and southwest Sulawesi, and *A. p. regalis* distributed in southeast Sulawesi (Kirwan et al., 2020a, 2020b; Eaton et al., 2021). Furthermore, both kingfishers are forest species that occupy different elevational ranges, where *A. monachus* occurs in lowland forests up to 1,300 m and is replaced by *A. princeps* at higher altitudes.

The high diversity of *Actenoides* makes this genus an ideal model for examining how Sulawesi's complex geological history has shaped phylogeographic patterns. We applied mitogenomic analysis to infer the phylogenetic relationship among *Actenoides* populations. The metagenomic approach to phylogenetic studies will provide more robust evidence of lineage differentiation than single locus inference. Therefore, our study could enlighten the biogeographic history of *Actenoides* in Sulawesi.

MATERIALS AND METHODS

2.1 Sample

We sequenced a total of 17 fresh samples covering *A. monachus* and *A. princeps* from most of their respective ranges (Fig. 1), and also included samples of *A. concretus* from Bangka Island and West Sumatra (Table 1).

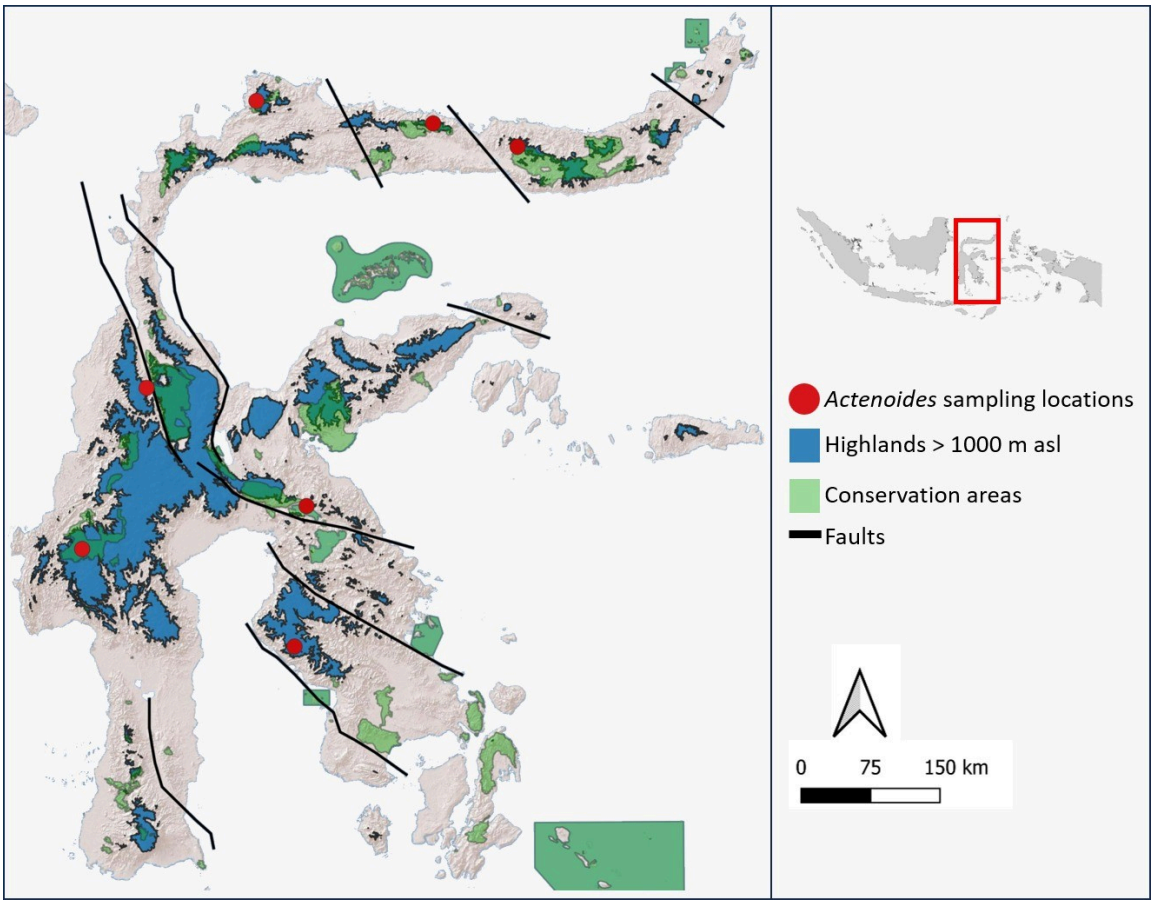

Figure 1. *Actenoides* sampling locations show most of the areas are within the highland of Sulawesi and spread across geological faults.

2.2 Laboratory Works

DNA extraction was performed using the MagMAX™ DNA Multi-Sample Ultra 2.0 Kit (CAT no. A36570) according to the manufacturer's protocol. Library preparation was carried out using the Tecan MagicPrep NGS system, utilizing Tecan cartridge-based reagents and pre-programmed scripts. Sequencing was performed on the Illumina platform at 10x coverage.

Table 1. List of samples used in this study

No.	Species	Location	Region	Numbers
1	*Actenoides princeps*	Mt. Gandang Dewata	Central Sulawesi	2
2		Mt. Dako	North Sulawesi	5
3		Mt. Boliyohuto	North Sulawesi	1
4		Mt. Torompupu	Central Sulawesi	1
5		Ilomata	North Sulawesi	1
6	*Actenoides monachus*	Ilomata	North Sulawesi	1
7		Mt. Mekongga	Southeast Sulawesi	2
8		Matano Lake	Central Sulawesi	2
9		Mt. Dako	North Sulawesi	2
10	*Actenoides concretus*	Mt. Talamau	West Sumatera	1
11		Mt. Maras	Bangka (Sumatra)	2
		TOTAL		**20**

2.3 Bioinformatics

2.3.1 Raw reads mapping

Raw reads were checked and cleaned using FastQC v0.11.7 (Babraham Bioinformatics) and Fastp v0.20.0 (Chen et al., 2018), and were rechecked with MultiQC (Ewels et al., 2016) after trimming. The clean raw reads were mapped against the complete mitochondrial genome reference of *Todiramphus sanctus vagans* (NC.011712.1) using BWA 0.7.17 and Samtools v1.6 (Li, 2013; Li et al., 2009). The NC.011712.1 genome was indexed using BWA 0.7.17 (Li, 2013) before being used as a reference genome. Fasta files were generated using ANGSD (Korneliussen et al., 2014) with -doFasta and quality scores set to 30. Mitochondrial genes were annotated using Mitos2 (Bernt et al., 2013).

2.3.2 Phylogenetic analysis

Fasta files were aligned using Mafft v7.526 (Katoh & Standley, 2013) with default options before running phylogenetic analysis. Phylogenetic reconstruction was conducted with maximum likelihood in IQTree applying ModelFinder and UFBoot (Kalyaanamoorthy et al., 2017; Hoang et al., 2018; Minh et al., 2020); while the Neighbour Joining tree was reconstructed in RStudio using ape package 5.8-1, in which genetic distances were calculated based on uncorrected p-distance (Paradis & Schliep, 2019). The maximum likelihood and neighbour joining trees were visualized using One Table (Xie et al., 2023). We added mitogenome of kingfishers from gene bank, i.e, *A. hombroni* SRR10185940, *A. lindsayi* SRR10186364, and *Todiramphus cinnamominus* SRR10294268, *T. sanctus* NC11712.1, *Halcyon senegalensis* NC52813.1, *H. smyrnensis* NC35746.1, *H. pileate* NC24198.1, *and H. coromanda* NC28177.1.

RESULTS

We reconstructed the phylogenetic relationships of kingfishers (*Actenoides*, *Todirhamphus*, and *Halcyon*) using approximately 18,000 bp. The best-fitting maximum-likelihood substitution model was GTR+F+I+G4, which showed some internal nodes were supported by high bootstrap values (≥95), indicating strong genetic divergence, particularly in the Ilomata population for both species (Fig. 2).

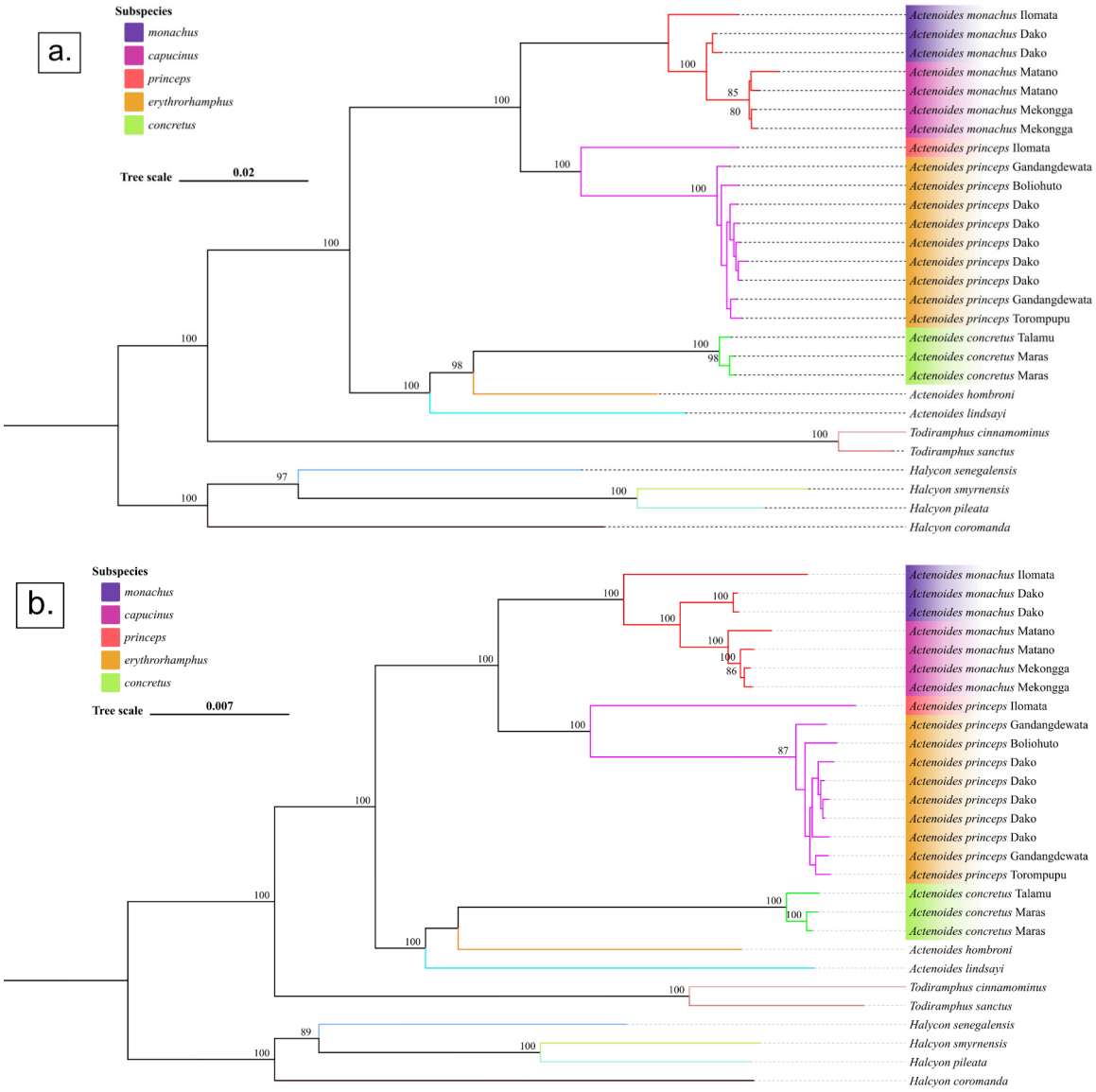

Figure 2. Phylogenetic Maximum Likelihood Tree (a) and Neighbor-Joining Tree (b) of *Actenoides* kingfisher.

The phylogenomic analyses consistently recovered three well-supported lineages corresponding to *A. concretus* (Sumatra), *A. monachus* (Sulawesi lowlands), and *A. princeps* (Sulawesi highlands). The neighbor-joining tree showed a similar pattern with the ML tree, and the genetic distance was >8% divergence between *A. concretus* and the Sulawesi taxa, as well as approximately 6% divergence between *A. monachus* and *A. princeps* (Fig 3).

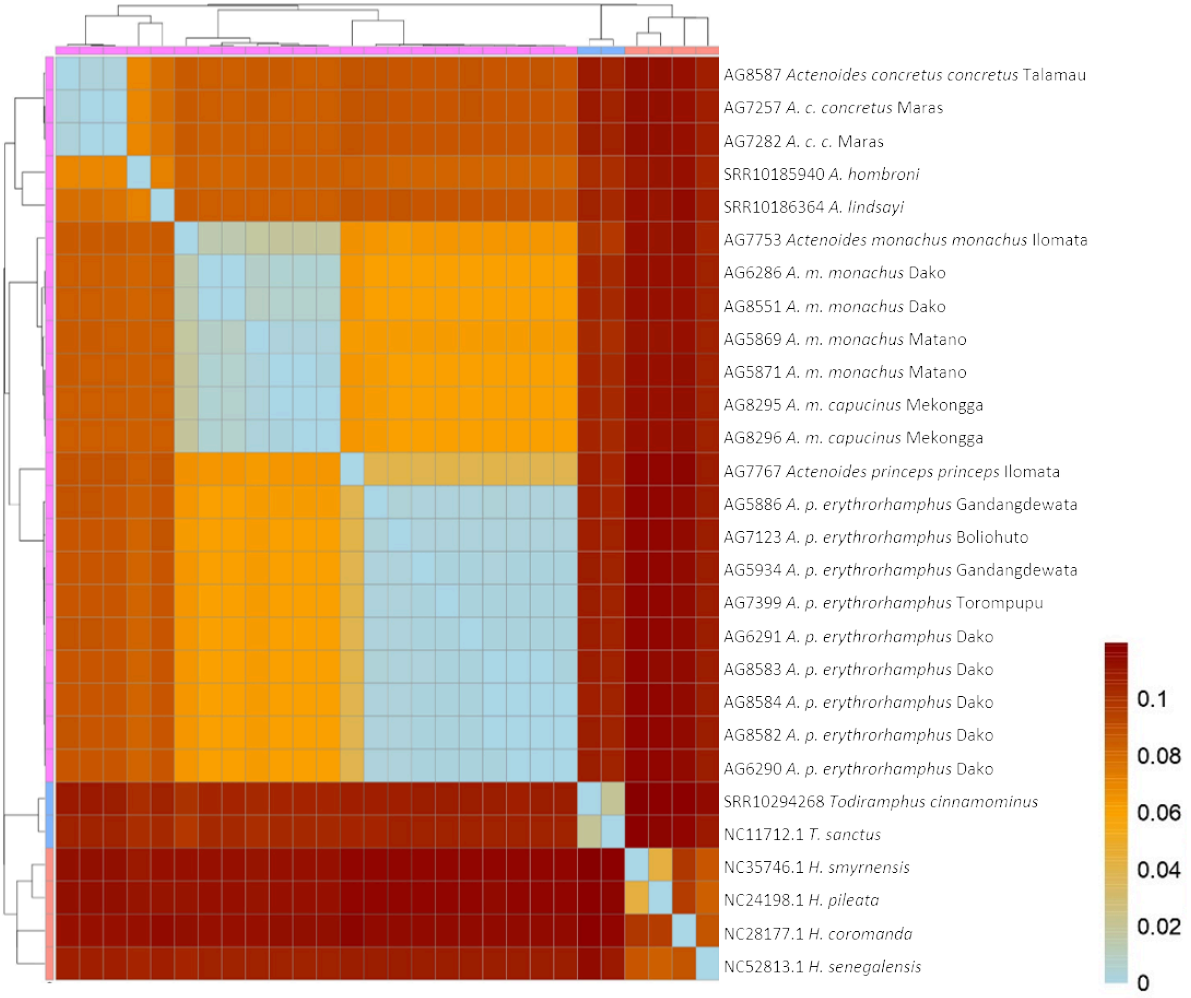

Figure 3. Heatmap of genetic distance based on uncorrected p-distance.

DISCUSSION

The phylogenetic tree reveals a clear divergence between the monophyletic clade of Sulawesi *Actenoides* and the clade consisting of *A. concretus*, *A. hombroni*, and *A. lindsayi*, where *A. concretus* is sister to the Philippine *Actenoides*. Multiple subclades are identified in each Sulawesi *Actenoides*, which are in accordance with subspecies delineation.

A. princeps from Ilomata is a sister to a combined clade of north-west and central populations and was positioned at the base of the *A. princeps,* suggesting that it represents a deeply divergent and long-isolated lineage. The population from Ilomata may represent the distribution limit of *A. princeps princeps* before being replaced by *A. princeps erythrorhamphus* as indicated by the phylogenetic trees.

A. monachus from Ilomata shows a similar pattern, which indicates distinct lineages from other clades. However, because the northern population of *A. monachus* shares similar morphological characters, it is treated as the same subspecies, *A. monachus monachus.*

Therefore, further study is needed to investigate whether phenotypical differences are present as shown by the phylogenetic analysis. In addition, population genomics insights may further reveal population structure among *A. monachus* populations to support the magnitude of divergence. Meanwhile, *A. monachus* from Matano forms the same clade as *A. monachus* from Mekongga, which is *A. monachus capucinus.*

Both *Actenoides* from Ilomata, where the population was located east of the Gorontalo fault, showed similar patterns of divergence, indicating the role of the Gorontalo Fault. While we did not analyze the timing of divergence, which could reveal a correlation with geological time, we expect the Gorontalo Fault to act as an ecological barrier to dispersal for both populations. There is a relatively wide, depressed lowland gap in the Gorontalo Fault that separates the montanus area from both directions, potentially preventing gene flow between the northwestern and northeastern populations. The pattern of divergence due to ecological barriers generated by faults could also occur in South Sulawesi populations. However, we could not confirm it in the present study due to the absence of samples from that region.

Our Sulawesi *Actenoides* pattern is identical to that of Sulawesi *Draco* (McGuire et al., 2023), which separates Illomata from other populations in northern Sulawesi. The research on Sulawesi *Draco* also noted several faults that formed during the island's formation. Our studies also reflect the biogeography of *Bufo celebensis*, particularly the separation between the north-central and northwest populations (Evans et al., 2003).

Our study provides new insights into the inland diversification of Sulawesi *Actenoides*, highlighting the influence of geological history on shaping their phylogeography. The faults created gaps between montane ranges, providing ecological discontinuity that reinforces geographical isolation, which further prevents gene flow between separated populations. The Tempe Depression in South Sulawesi is one of the extraordinary features that support the argument that fault-driven topographic fragmentation shaped the Sulawesi phylogeographic structure, which explains that there are several species and subspecies that are only found in the Lompobattang montane complex (Eldridge et al., 2018; Evans et al., 2003; McGuire et al., 2023).

Our findings support the hypothesis that Sulawesi's geological and environmental history has driven inland diversification within endemic avifauna, following patterns consistent with vicariance, geographic isolation, and montane habitat specialization (Rahbek et al., 2019; Stelbrink et al., 2012). These results also showed that montane environments in Sulawesi play a significant role in the formation of endemicity in the region. Further integrative studies

combining ecological, behavioral, and genomic data are recommended to refine our understanding of the mechanisms of speciation and the historical biogeography that have shaped Sulawesi's exceptional biodiversity.

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