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## Short Note

# Genetic divergence of the Sunda ratsnake (*Coelognathus subradiatus*) across the Lesser Sunda Islands (Squamata: Colubridae)

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**Abstract.** The Lesser Sunda Archipelago consists of hundreds of oceanic islands located in southern Wallacea. The Sunda ratsnake, *Coelognathus subradiatus*, is endemic to the Lesser Sundas and is found on most of the major islands. Mitochondrial DNA was sequenced from snakes representing five of the major islands revealing that levels of sequence divergence between islands range from 2-7%. Phylogenetic analyses recover what can be interpreted as a three-lineage polytomy consisting of lineages from 1) Alor, 2) Sumbawa + Flores, and 3) Timor + Wetar. The archipelago was colonized from the Sunda Shelf ~7 Myr with subsequent population divergence occurring ~4.5 Myr, likely resulting in insular species formation.

**Keywords:** biogeography, Indonesia, mitochondrial DNA, phylogeography, reptiles.

Alfred Russel Wallace's observations of faunal distributions in the Lesser Sunda Islands helped inspire his formation of the field of biogeography (Wallace, 1860). These islands are relatively old and have never been connected to a continental shelf, suggesting that terrestrial taxa colonized the archipelago by swimming or rafting. The oldest islands in the archipelago (continuously emergent since ~8-12 Myr) are those of the eastern portion of the volcanic Sunda Arc which include Lombok, Sumbawa, and Flores (fig. 1a; Hall, 2009, 2011). The volcanic islands of the Inner Banda

Arc (e.g., Alor, Wetar) are estimated to have been continuously emergent since ~5 Myr, and the non-volcanic Outer Banda Arc (e.g. Rote, Timor) and Sumba continuously emergent since ~2-3 Myr (fig. 1a; Hall, 2009, 2011). Some of these islands are thought to periodically become connected by land-bridges (e.g., Lombok + Sumbawa, Komodo + Flores + Lembata) during glacial maxima when sea levels are up to 120 m lower (Bintanja, van de Wal, and Oerlemans, 2005).

While the higher-level taxonomic diversity of amphibians and reptiles within the archipelago is generally well documented, there are a number of widespread taxa that occur on islands or island-sets that are separated by oceanic barriers that have existed for millions of years. Some of these species that entered the archipelago early on have diversified and now represent species complexes (Reilly, 2016; Blom et al., 2019; Reilly et al., 2019a), while others have recently arrived and rapidly expanded through the archipelago (Reilly et al., 2017, 2019b). Here we examine the Sunda ratsnake (*Coelognathus subradiatus*), one of eight species of

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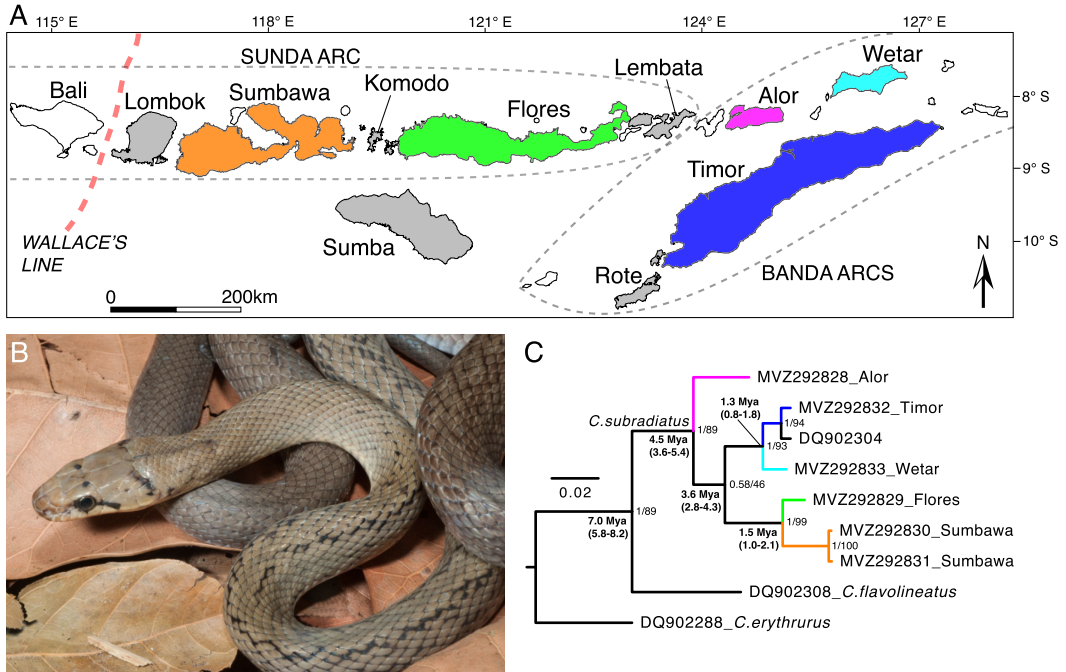
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**Figure 1.** (A) Map of the Lesser Sunda region and Bali. Sampled islands are colored and unsampled islands that are inhabited by *Coelognathus subradiatus* are shaded in light gray. (B) *Coelognathus subradiatus* from Alor Island (Photo: J. McGuire). (C) Bayesian phylogeny of the *ND4* gene. Numbers to the right of nodes represent posterior probability/bootstrap support, and bold numbers below nodes represent estimated divergence times in millions of years (Mya) with 95% posterior density values in parentheses.

snake endemic to the Lesser Sundas (fig. 1b). There is substantial morphological variation between populations on different islands or island groups that remained isolated during glacial maxima (How, Schmitt, and Suyanto, 1996), and it has been suggested that the species is in need of taxonomic revision (De Lang, 2011). In this study we examine mitochondrial data from seven specimens representing the islands of Sumbawa, Flores, Alor, Wetar, and Timor, to determine the level of genetic divergence and phylogenetic relationships between island populations.

Samples of *Coelognathus subradiatus* were collected on field expeditions to the islands of Sumbawa, Flores, Alor, Wetar, and Timor (table 1). Genomic DNA was extracted from liver tissue using the Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). An ~890 bp portion of the mitochondrial

*ND4* gene, tRNA-His, tRNA-Ser, and tRNA-Leu was PCR amplified using the primers ND4 and LEU (Arévalo et al., 1994). PCR products were cleaned using ExoSAP-IT (USB, Cleveland, OH, USA) before being used in cycle sequencing reactions using BigDye v3.1 (Applied Biosystems, Foster City, CA, USA) with both forward and reverse primers. Cycle sequencing products were ethanol precipitated and sequenced on an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). Forward and reverse reads were combined in GENEIOUS v11.1.5 (<https://www.geneious.com>) and aligned with existing GenBank sequences from *Coelognathus subradiatus* (DQ902304-locality unknown), *C. flavolineatus* (DQ902308), and *C. erythrurus* (DQ902288) using MUSCLE (Edgar, 2004). The software MODELGENERATOR (Keane et al., 2006) selected HKY+G as the best-fit nucleotide substitution model which was used in all phylogenetic

**Table 1.** Sample locality information and GenBank numbers.

Museum #	Island	Verbatim locality	Latitude	Longitude	<i>ND4</i> GenBank
MVZ292828	Alor	Desa Waisika, Kecamatan Alor Timor Laut, Kabupaten Alor, Provinsi Nusa Tenggara Timur	-8.18916	124.75098	MT524074
MVZ292829	Flores	Desa Mataloko, Kecamatan Golewa, Kabupaten Ngada, Provinsi Nusa Tenggara Timur	-8.82344	121.04986	MT524075
MVZ292830	Sumbawa	Desa Bolo, Kecamatan Bolo, Kabupaten Bima, Provinsi Nusa Tenggara Barat	-8.51034	118.59798	MT524076
MVZ292831	Sumbawa	Desa Tolotangga, Kecamatan Monta, Kabupaten Bima, Provinsi Nusa Tenggara Barat	-8.76643	118.60496	MT524077
MVZ292832	Timor	Desa Camplung Dua, Kecamatan Fatuleu, Kabupaten Kupang, Provinsi Nusa Tenggara Timur	-10.02378	123.86585	MT524078
MVZ292833	Wetar	Desa Ilwaki, Kecamatan Wetar Selatan, Kabupaten Maluku Barat Daya, Provinsi Maluku	-7.92627	126.40800	MT524079

analyses. Maximum Likelihood (ML) phylogenetic analysis was performed using the program RAXML v8 (Stamatakis, 2014) with 1000 non-parametric bootstrap replicates to assess node support. Bayesian phylogenetic estimation was carried out using the software MRBAYES v3.2 (Ronquist et al., 2011) and run for 10 million generations. After the standard deviation of split frequencies was confirmed to be well below 0.01, a burnin of 25% was removed before summarizing the remaining trees as a phylogram. Divergence times were estimated using BEAST v2.4 (Bouckaert et al., 2014) under a strict molecular clock with a rate of 1.7% divergence per million years as estimated for the *ND4* gene from the bovid species *Chilabothrus monensis* (Rodríguez-Robles et al., 2015). After all ESS values were confirmed to be >200 using TRACER v1.7 (Rambaut et al., 2018), two runs of 50 million generations were combined after a burnin of 10% from each to create a maximum clade credibility tree. The uncorrected percent sequence divergence between lineages was estimated using the software DNASP V5 (Librado and Rozas, 2009).

The final sequence alignment consisted of 892 bp and all new sequences have been deposited in GenBank (table 1). The tree was rooted with *C. erythrurus* following the phylogeny estimated by Burbrink and Lawson (2007). Both ML and Bayesian phylogenies recovered the same tree topology containing a monophyletic *C. subradiatus* (PP = 1; BS =

89), with low support for the clade containing Sumbawa + Flores + Wetar + Timor (PP = 0.58; BS = 46), though the BEAST analysis has better support (PP = 0.94) for this clade. Well supported sister relationships were obtained for Timor + Wetar (PP = 1; BS = 93) and Sumbawa + Flores (PP = 1; BS = 99). The *C. subradiatus* specimen in GenBank with an unknown locality is sister to the Timor sample (PP = 1; BS = 94), suggesting it was originally obtained from Timor or an adjacent island such as Rote. The divergence between *C. subradiatus* and its sister species *C. flavolineatus* is estimated at  $\sim 7.0$  ( $\pm 1.2$ ) Mya. Initial lineage divergence began  $\sim 4.5$  ( $\pm 0.9$ ) Mya with the split between the Timor + Wetar and Flores + Sumbawa clades occurring  $\sim 3.6$  ( $\pm 0.8$ ) Mya. Flores and Sumbawa diverged  $\sim 1.5$  ( $\pm 0.5$ ) Mya and Timor and Wetar diverged  $\sim 1.3$  ( $\pm 0.5$ ) Mya. Island lineages range in divergence between 2-7%, and the unknown locality sample is 0.7% divergent from our Timor sample (table 2).

The genetic structuring in this system suggests that *C. subradiatus* may represent a

**Table 2.** Average number of substitutions per site between island lineages of *Coelognathus subradiatus*.

	Flores	Alor	Wetar	Timor
Sumbawa	0.025	0.071	0.059	0.061
Flores	-	0.064	0.055	0.055
Alor	-	-	0.056	0.053
Wetar	-	-	-	0.020

species complex, which has been suggested previously on the basis of morphology (How, Schmitt, and Suyanto, 1996). For example, there are three clades that are each greater than 5% sequence divergent from one another: the Alor sample, a clade composed of populations on the Sunda Arc islands of Sumbawa and Flores, and a clade composed of samples from Timor and Wetar. Our genetic groupings are similar but not identical to the morphology-based groupings suggested by How, Schmitt, and Suyanto (1996) on the basis of head scalation and body measurements, with the only difference being that our data indicate that Timor and Wetar are sister lineages whereas How, Schmitt, and Suyanto (1996) proposed that the Wetar population should be grouped with that of its neighboring island of Alor. Determining whether these clades should ultimately be formally recognized as species must await more complete sampling (particularly from major islands such as Lombok and Sumba), as well as multilocus genetic data that would be better suited to estimate relative gene flow between these putative lineages.

Due to the low support for the clade containing Sumbawa, Flores, Wetar, and Timor our results can be interpreted as a three-lineage polytomy for our *C. subradiatus* samples. However, we can speculate on the sequence of colonization events given our time-calibrated phylogenetic estimate together with age estimates for the islands. *Coelognathus subradiatus* and its sister species *C. flavolineatus* (which occurs on the Sunda Shelf, including Bali) diverged ~7 Mya representing the initial colonization of the Lesser Sundas by overwater dispersal across Wallace's Line. The inferred timing of this split is after the major Sunda Arc islands had emerged but before either the Inner Banda Arc or Outer Banda Arc islands had yet arisen (Hall, 2009, 2011). The Inner Banda Arc island of Alor, for example, is about 5 million years old (Hall, 2009). This combination of lineage and island ages suggest that the common ancestor of *C. subradiatus* did not first reach Alor

when colonizing the Lesser Sundas despite that the Alor lineage is inferred (with modest support) as the sister taxon of the remainder of the complex. Rather, it suggests that a major Sunda Arc island such as Lombok, Sumbawa, or Flores may have been first colonized ~7 Mya, with one of these islands serving as a springboard into the Banda Arcs at ~4.5 Mya. For some other taxa, the colonization of the outer Banda Arc Islands involved initial dispersal to Sumba from the Sunda Arc followed by dispersal from Sumba to Timor (Reilly, 2016), but we can only speculate about this possibility here because we lack sampling from Sumba. Additionally, colonization of the volcanic Inner Banda Arc islands (Pantar, Alor, and Wetar) from Timor, rather than by way of the more proximal Sunda Arc islands (Flores, Lembata, etc.), is a repeated scenario in taxa that colonized the archipelago from Asia early on (Reilly, 2016, unpubl. data), which suggests that Wetar may have been colonized from Timor in this case.

More genetic data are needed to confirm the phylogenetic relationships of other populations including the unsampled islands of Lombok, Sumba, Komodo, Rinca, Adonara, Lembata, Semau, and Rote. Additionally, *C. enganensis* from Enggano Island (off of the southwest coast of Sumatra) was once considered a subspecies of *C. subradiatus* yet its phylogenetic relationship to other members of the genus are not currently known (Brongersma, 1934; Schulz, 1996).

While this study is limited in scope with respect to number of samples and genes, it has revealed that *C. subradiatus* entered the archipelago after the Sunda Arc islands became emergent but before the Banda Arc Islands existed. Our data suggest at least three divergent populations exist that have been isolated for a substantial amount of time. This evidence, along with the relationships of island lineages, adds to the growing body of evidence suggesting that early reptile and amphibian colonizers of the Lesser Sundas have experienced a complex biogeographical history and that the

oceanic barriers between islands have resulted in insular species formation.

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