

# Preliminary studies of cryptic species: Phylogenetic Relationship of *Cyrtodactylus* sp. from Lombok Island using the NADH Dehydrogenase Subunit 2 gene

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**Abstract.** Genus *Cyrtodactylus* has high morphological diversity, which often leads to confusion in its classification. The geographical history formed from ocean islands and were not separately part of the continental shelf. This research was conducted to confirm the identity of the *Cyrtodactylus* sp. specimen from Lombok Island using ND2 gene, taken from 5 different locations. Molecular analysis was carried out through the stages of DNA extraction from liver tissue using the NexPrep™ Cell/Tissue DNA Mini Kit, DNA quality testing by electrophoresis, target gene amplification using the Polymerase Chain Reaction method with Primers M112F and M1123R. The results of alignment are 600 bp, BLAST and phylogenetic analysis results confirmed that three specimens of *Cyrtodactylus* sp. from Lombok Island are the same species as *C. jantai* from Bali Island. Hopefully, this research can help develop similar research and future map conservation strategies for the *Cyrtodactylus* spp.

Keyword: *Cyrtodactylus*, ND2, Lombok Island

## 1 Introduction

*Cyrtodactylus* is a group of fauna from the Reptile Class Gekkonidae Family [1]. *Cyrtodactylus* is spread across Asia, especially Southeast Asia, the Pacific Islands, and Australia. This genus is commonly known as curved-finger lizards, bent-finger lizards, or rock lizards and is the most diverse group of lizards. There are 259 recorded member species, which is still increasing; *Cyrtodactylus* is the largest genus in the Gekkonidae family [2]. *Cyrtodactylus* is a complex cryptic species with an abundant population and widespread in

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Indonesia [3]. A cryptic species is two or more different species that are classified as a single species because of their morphological similarities [4]. Molecular approaches in the study of reptile taxonomy have proven effective in revealing the identity of a species that is morphologically difficult to distinguish [5].

Molecular biology techniques can help the sustainability of the biological world, which is increasingly threatened. The lack of genetic information means species are feared to become extinct before being recognized. The effectiveness of molecular biology systems and techniques is significant for conserving the genetic resources of a species with a high level of accuracy in efforts to preserve living things. Methods for identifying living species have developed from morphological to molecular identification based on short pieces of DNA called DNA barcodes [6]. DNA barcode is a species identification technique; its application is similar to barcode scanning technology in commercial products. The NADH Dehydrogenase Subunit 2 (ND2) gene is the protein-coding gene used for species identification.

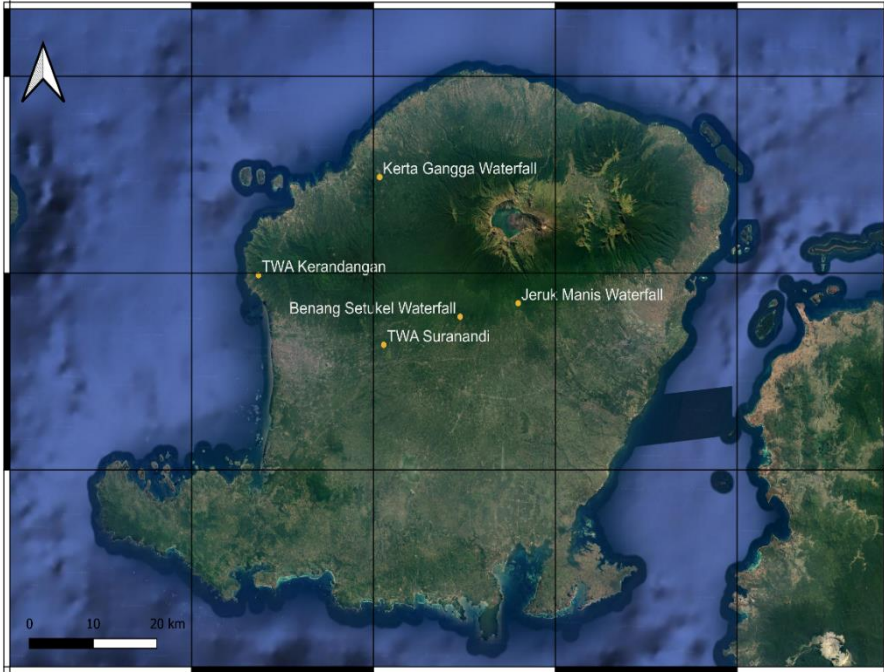
The ND2 gene was chosen because, generally, this gene has become the standard for determining species groups in the genus *Cyrtodactylus* and consistently obtains accurate values in several nodes [7]. In addition, ND2 can produce a group of mitochondrial enzymes (mtDNA). Genes from mitochondrial DNA are widely used because mtDNA inheritance is maternal [8]. Reconstruction of taxa using the ND2 gene can also interpret the divergence, variation, and phylogenetics of living things related to their biogeographic history [9]. Changes located in the nucleotide base sequence in the ND2 gene can occur due to geological separation, resulting in habitat changes. ND2 is also used to determine the level of genetic diversity of an individual, population, and animal species [10].

Previous studies confirmed that the *Cyrtodactylus* sp. population on Lombok Island has several different species, namely *C. gordongekkoi* and *C. darmandvillei*. Young volcanic areas dominate Lombok Island and only emerged during the miocene to the end of the paleocene. The island appeared to form an arc around 12-1 million years ago. This region is included in the wallacea region. If we look at previous research, wallacea is still an area that has not been studied much regarding the diversity of *Cyrtodactylus* because of the large number of small islands and the challenge of conducting field surveys in each region [11]. The geographical conditions of Lombok Island can result in adaptation to geographic barriers, resulting in the discovery of various morphological species with certain key characters. Therefore, this study aims to confirm the species identity and phylogenetic of *Cyrtodactylus* spp. samples from Lombok Island based on the ND2 gene.

## 2 Research Methods

### 2.1 Time and Location of Research

The research conducted from January to March 2024. *Cyrtodactylus* spp. samples were taken from 5 different locations on Lombok Island (Figure 1). Sampling was taken using the VES method [12]. Molecular analysis was conducted in collaboration with the Animal Systematics Laboratory, Faculty of Biology, Gadjah Mada University.



**Figure 1.** Location of sample collection for the genus *Cyrtodactylus* in Lombok Island (Visualized using QGIS)

## 2.2 DNA Extraction, Quality Testing, and Quantity

DNA extraction from 10 mg of liver tissue using the NexPrep™ Cell/Tissue DNA Mini Kit. DNA quality testing uses the electrophoresis method [13]. After obtaining high concentrations of DNA, the next step is amplification of the ND2 target gene using reference primers.

## 2.3 Amplification and Sequencing of the ND2 Gene

Then, using the Biorad PCR tool, 15 µl of Nexpro PCR Master Mix, three µl of template DNA sample (100 ng/µl), six µl of DDH<sub>2</sub>O, and 3 µl of primer (forward and reverse primer each). The primers used were M1123F (5'-AAGCTT TCGGGCCCCATACC-3') and M1123R (5'-GCTTAATTAAGTGTYTGAGTTGC -3').

Amplification is carried out by setting the temperature as follows: predenaturation at 94 °C for 9 min, followed by denaturation at 94 °C for 45 s, annealing at 60.1 °C for 45 s, and 1 min at 72 °C consisting of extension. Next, post-extension treatment was carried out at 72°C for 7 minutes. The PCR results were then electrophoresed on 1% agarose. The PCR results were then sequenced using 1st BASE Laboratories Sdn Bhd sequencing services.

## 2.4 Phylogenetic Reconstruction

Contig sequencing results using the GenStudio application. The sample sequence of *Cyrtodactylus* spp. from Lombok Island is compared with *Cyrtodactylus* from the same complex in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Data analysis using NCBI BLAST,

uncorrected p-distance calculations, and maximum likelihood phylogenetic tree reconstruction using MEGA 11 [14].

### 3 Results and Discussion

#### 3.1 Homology Identification

Table 1. Sequence homology of *Cyrtodactylus* samples with sequences *Cyrtodactylus* GenBank

	Blast NCBI		
Sample	Identification	%	Seq ID
Benang Stokel, Central Lombok	<i>Cyrtodactylus jatnai</i>	99.08	OP356362.1
Jeruk Manis, East Lombok	<i>Cyrtodactylus gordongekkoi</i>	99.40	OP356352.1
Krandangan, West Lombok	<i>Cyrtodactylus jatnai</i>	99.65	OP356361.1
Suranandi West, Lombok	<i>Cyrtodactylus jatnai</i>	99.33	OP356362.1
Karta Gangga, North Lombok	<i>Cyrtodactylus gordongekkoi</i>	99.51	OP356352.1

Sequence alignment of *Cyrtodactylus* samples from Lombok showed that three samples had high homology with *Cyrtodactylus* sequences *C. jatnai* from Bali, namely around 99.08% (Benang Stokel), 99.65% (Krandangan), and 99.33% (Suranandi). In comparison, the other two samples have high homology with the *Cyrtodactylus gordongekkoi* sequence from Lombok, namely around 99.40% (Jeruk Orange) and 99.51% (Karta Ganga). Interpretation of the similarity of sample sequences and database sequences is stated as follows: sequence similarity of 97-100% is said to be significant, 92-96% is quite significant, while similarity <91% is said to be insignificant [15,16]. High homology indicates that most of the specimen's genetic material is in identical individuals (GenBank).

The use of the ND2 gene, which is part of mtDNA, is the right choice because mtDNA is maternal. Therefore, *Cyrtodactylus* specimens that are related by maternal inheritance will have similar sequences, while those not related by maternal inheritance will show different sequences. ND2 determines the genetic diversity level in individuals, populations, and animal species [10]. Although the ND2 gene is not for animal barcodes, it can be used for identification because ND2 has the appropriate phylogenetic reconstruction of *Cyrtodactylus* with COI DNA barcoding [7]. In phylogenetic analyses of the genus *Cyrtodactylus*, ND2 is most commonly used if using other genes would complicate genetic identification [1].

#### 3.2 Genetic Distance

Table 2. Uncorrected p-distances (in %) for ND2 gene fragment (600 bp) among *Cyrtodactylus* spp. (*C. sadleiri* complex) taxa compared in this study.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Benang Stokel, Central Lombok	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

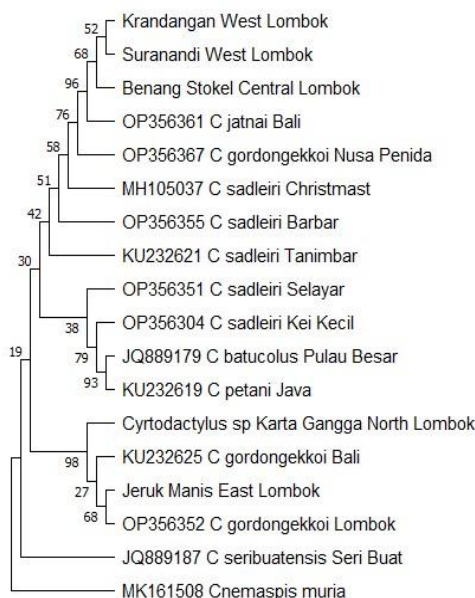
Jeruk Manis, East Lombok	8.45 %	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Krandangan, West Lombok	0.17 %	8.28 %	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Suranandi_West_Lombok	0.17 %	8.28 %	0.00 %	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Karta Gangga, North Lombok	8.28 %	0.17 %	8.11 %	8.11 %	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. batucolus</i> Pulau Besar	11.32 %	10.81 %	11.15 %	11.15 %	10.64 %	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. seribuatenensis</i> Seri Buat	9.12 %	1.52 %	8.95 %	8.95 %	1.35 %	11.15 %	-	-	-	-	-	-	-	-	-	-	-
<i>C. petani</i> Java	10.98 %	10.30 %	10.81 %	10.81 %	10.14 %	6.25 %	10.81 %	-	-	-	-	-	-	-	-	-	-
<i>C. sadleiri</i> _Tanimbar	6.59 %	6.42 %	6.42 %	6.42 %	6.25 %	10.81 %	7.26 %	10.81 %	-	-	-	-	-	-	-	-	-
<i>C. gordongekko</i> Bali	8.29 %	0.17 %	8.12 %	8.12 %	0.00 %	10.66 %	1.35 %	10.15 %	6.26 %	-	-	-	-	-	-	-	-
<i>C. sadleiri</i> Christmas	4.22 %	7.94 %	4.05 %	4.05 %	7.77 %	10.30 %	8.78 %	10.98 %	6.25 %	7.78 %	-	-	-	-	-	-	-
<i>C. sadleiri</i> Kei Kecil	8.95 %	9.63 %	8.78 %	8.78 %	9.46 %	9.63 %	10.47 %	9.29 %	9.29 %	9.48 %	8.61 %	-	-	-	-	-	-
<i>C. sadleiri</i> Selayar	9.63 %	9.12 %	9.80 %	9.80 %	8.95 %	12.50 %	9.97 %	11.66 %	9.80 %	8.97 %	9.46 %	9.29 %	-	-	-	-	-
<i>C. gordongekko</i> Lombok	8.45 %	0.00 %	8.28 %	8.28 %	0.17 %	10.81 %	1.52 %	10.30 %	6.42 %	0.17 %	7.94 %	9.63 %	9.12 %	-	-	-	-
<i>C. sadleiri</i> Barbar	6.25 %	8.11 %	6.08 %	6.08 %	7.94 %	9.63 %	8.61 %	9.63 %	6.25 %	7.95 %	5.74 %	8.45 %	9.97 %	8.11 %	-	-	-
<i>C. jatnai</i> Bali	0.34 %	8.11 %	0.17 %	0.17 %	7.94 %	9.98 %	9.12 %	10.64 %	6.25 %	7.95 %	4.22 %	8.61 %	9.63 %	8.11 %	5.91 %	-	-
<i>C. gordongekko</i> Nusa Penida	4.39 %	8.95 %	4.22 %	4.22 %	8.78 %	11.49 %	9.63 %	11.82 %	7.94 %	8.80 %	4.90 %	10.47 %	10.81 %	8.95 %	6.25 %	4.05 %	-
<i>H. frenatus</i> Pahang	33.05 %	33.78 %	32.88 %	32.88 %	33.15 %	32.50 %	33.00 %	32.20 %	33.60 %	33.24 %	32.43 %	33.83 %	35.00 %	33.70 %	34.20 %	32.88 %	33.50 %

Table 2 shows the p distance without correction for the Lombok Island sample. *Cyrtodactylus gordongekko* is a type of *cyrtodactylus* previously discovered on the island of Lombok [17]. *C. gordongekko* is one of the species of the *C. sadleiri* complex. For example,

*Cyrtodactylus* from sweet orange has a close genetic distance to *Cyrtodactylus gordongekkoi*, with 0.00% for Jeruk Manis and 0.17% for Karta Gangga. Three other samples showed relatively high genetic distances, respectively: Benang Stokel (8.45%), Krandangan (8.22), and Suranandi (8.22). These three samples are closer to *C. jatnai* from Lombok, respectively Benang Stokel (0.34%), Krandangan (0.17%), and Suranandi (0.17%). Consequently, additional morphological studies comparing type specimens must confirm their status.

The level of genetic divergence shown by genetic distance can help find hidden species [18]. Interspecies and intraspecies relationships can be identified using genetic distance values. High genetic distance makes identification at the family or genus level easier [19]. Genetic distance variation can be influenced by overexploitation and environmental factors [20]. In addition, population phylogenetics and morphology can change due to geographic location and environmental conditions [21].

### 3.3 Phylogenetic Tree



**Figure 2.** Maximum Likelihood tree of ND2 alignment showing phylogenetic relationship *Cyrtodactylus* spp. from Lombok Island.

The results of the phylogenetic reconstruction analysis are depicted in the phylogeny tree (Figure 2). Specimens from Krandangan, Benang Stokel, and Suranandi occupy maximum likelihood branches with *C. jatnai* from Bali, with a confidence value of 96%. The results of the phylogenetic reconstruction show that the *Cyrtodactylus* specimens from Jeruk Manis and Karta Gangga are more closely related to *Cyrtodactylus gordongekkoi* from Lombok with a bootstrap value of 98%.

Stable clades have bootstrap values of 95 percent or more [22]. Phylogeny studies the evolutionary relationships between groups of organisms and creates hypotheses about how they relate to each other and their history [23,24]. The predicted relationships between taxa and their hypothesized common ancestor are depicted visually in a phylogenetic tree [25]. Consequently, bootstrap analysis was used to evaluate the homogeneity of clusters in the phylogenetic tree and validate the clusters in the branch arrangement [26]. In addition,

possible species relationships in a phylogenetic tree can be identified more accurately by counting the number of repetitions in the sequence alignment using the bootstrap method

## 4 Conclusion

The alleged discovery of new locality records of *Cyrtodactylus jatnai* in Lombok (Suranandi, Krandangan, and Benang Stokel) and discovery of cryptic species in two populations of the *Cyrtodactylus darmandvillei* complex. The habitat of *Cyrtodactylus* on Lombok Island is found at an altitude of around 50-600 meters above sea level in karst areas and secondary forests. However, morphological research is needed to confirm the taxonomic status of the endemic and poorly known genus *Cyrtodactylus* in Lombok Island

## 5 Acknowledgements

We want to thank the West Nusa Tenggara Natural Resources Conservation Center for Mount Rinjani National Park for issuing permits to access, survey, and collect specimens from the area. This research was supported by the synergistic efforts of the "Herpetology Study Club" who helped carry out sampling in the field. This research was financially supported by the Indonesia Endowment Fund for Education Agency (LPDP).

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