Classification of *Cyrtodactylus marmoratus* species complex based on the molecular approach

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Abstract. Sundaland’s construction history has implications for the diversification and distribution of the genus Cyrtodactylus. One of the Cyrtodactylus known is *C. marmoratus* which taxonomically uncleared species. The aim of this study was to explain the taxon position of *C. marmoratus* from Java using the ND4 gene marker. Total of 10 Cyrtodactylus were analyzed based on the molecular analysis. Afterwards, it was used to construct the phylogenetic trees based on the maximum likelihood, maximum parsimony and Bayesian Interference. The p-distance analysis was tested to determine the differences between species. Interestingly, the phylogenetic trees indicated that the *C. marmoratus* from West Java was separated from *C. marmoratus* from Central Java. Some *C. marmoratus* from central Java were grouped with *C. semiadii*. It was also supported by the morphological characters of *C. marmoratus* from West Java which has distinguishing features i.e. no pore on precloacal scales, no continuous pore on precloacal femoral scale, no deep precloacal groove, have 37 ventral scales and 20 lamellae sub-digital on the fourth toe. Thus, it can be confirmed that *C. marmoratus* was a species complex with unclear distribution limits.

1. Introduction

The complexity construction of Sundaland was caused by the process of merging and separating of the islands in the region due to the decrease and increase of the sea level during the Pleistocene and Pliocene periods. The Sundaland was formed due to the expansion of the Asian mainland during the period of low sea level and the rise of the seabed between Semenanjung Malaya, Java, Sumatra and Kalimantan [1]. The construction process influences the genetic distribution from the mainland to the small islands [2]. The emergence of a geographical barrier in the Sundaland region initiates the speciation process [3]. This condition was also implicating the Cyrtodactylus genus. The Cyrtodactylus genus has experienced an increase in the number of new species based on the morphological and molecular characterization in the previous study [4]. The number of new species occurred due to the adaptation of Cyrtodactylus towards the limestone habitats and others [4,5].

Cyrtodactylus is a genus of Gekkonidae with the most number of species (232 species). Total of 232 species was distributed from Asia to Australia [6]. The distribution is very wide in almost all of the Southeast Asia. The different species of Cyrtodactylus was usually characterized based on the morphology due to the adaptation towards the geographical barriers. *C. marmoratus* is a species that belongs to a species complex which can be found in the Java island. However, its type locality still remains unknown [7]. Thus, it is necessary to identify *C. marmoratus* based on the molecular approach. This study aimed to explain the taxon position of *C. marmoratus* based on its ND4 gene sequences.
2. Materials and Methods

A total of 10 Cyrtodactylus were collected during herpetological surveys in Java and Sumatra on 2013 and 2014 (Table 1.) Live samples of Cyrtodactylus were photographed on manipulating studio photos to make it similar to their natural habitat. The photo was taken from the dorsal, lateral and ventral of the Cyrtodactylus’s body. Tissue samples were taken from the liver organ or muscle and stored in the 1.5 ml tubes containing absolute ethanol 96-100%. The died samples were preserved using 4% formalin then transferred to a barrel containing 70% ethanol.

2.1 Molecular methods

The DNA was extracted using the Qiagen amp DNA extraction kit. The ND4 gene was amplified using “ND4” marker i.e. forward primer (5’-TGA CTA CCA AAA GCT CATTLE GAA GC- 3’) and reverse primer (5’-TA CCT TTA CTT GGA TTT GCA CCA-3’). The ND4 gene amplification was carried out on the total of 40 µl reaction with the following thermal cycling PCR cycles: pre-denaturation step 93ºC for 3 min, followed by 32 cycles of denaturation at 93ºC for 30s, annealing at 60 ºC for 45s and extension at 72 ºC for 1 min. The last cycle was followed with the final extension at 72 ºC for 7 min. The amplicons were visualized on 1.5% agarose gel electrophoresis to confirm the DNA bands and molecular weight. Then, it was sequenced by 1st BASE Malaysia.

Table 1. Specimens localities and GenBank Accesion number.

| Species          | Locality                     | GenBank Accesion No. |
|------------------|------------------------------|----------------------|
| \textit{C. marmoratus} | Sukabumi, Jawa Barat         |                      |
| \textit{C. marmoratus} | Nusakambangan, Jawa Tengah  |                      |
| \textit{C. semiadii} | Cilacap, Jawa Tengah        |                      |
| \textit{C. cf psarops} | Karo, Sumatra Utara         |                      |
| \textit{C. cf psarops} | Tapanuli, Sumatra Utara     |                      |
| \textit{C. cf psarops} | Payakumbuh, Sumatra Barat   |                      |
| \textit{C. semicinctus} | Kerinci, Jambi              |                      |
| \textit{C. lateralis} | Seulawah Agam, Aceh         |                      |
| \textit{C. consobrinus} | Andalas, Sumatra Barat      | EU268412.1           |
| \textit{C. cf psarops} | Samosir, Sumatra Utara      |                      |
| \textit{C. consobrinus} | Kalimantan Barat, Kalimantan| EU268413.1           |
| \textit{C. loriae} | Papua Nugini                | EU268422.1           |
| \textit{Gekko gecko} | China                       |                      |
| \textit{Cnemaspis limi} | Malaysia                    | NC020039.1           |

2.2 Phylogenetic construction

Forward and reverse sequences were edited using Sequencer software. It was aligned using clustalW from MEGA 7.0 software. The phylogenetic tree construction was carried out based on the Maximum likelihood (ML), Maximum Parsimony (MP) and Bayesian Interference (BI). The model test of ML was conducted using a J model test based on Akaike Information Centre (AIC). Whereas, the evolution model was analyzed using Bayesian Interference from Kakusan 4 software. Maximum likelihood and Maximum Parsimony were analyzed using the PAUP * 4.0 software [8]. The modelling of the model test calculation from Maximum likelihood based on AIC obtained the most suitable evolution model was TrN + I + G. The calculation of phylogenetic tree branches based on MP was carried out using Heuristic search options. It was analyzed by 10x replications of Tree Bisection recognition (TBR) with 1000x bootstraps. The Bayesian interference was carried out using Mr.Bayes 3.0b software by calculating the posterior Bayesian probabilities using MCMC estimation with parameter retrieval frequency performed every 1000 generations. The calculation was completed if the standard deviation value has reached less than 0.01. The bootstrap values were considered valid when it reaches more than 70% for ML and MP, while Bayesian Interference was more than 95%.
3. Result and Discussion

Total of 12 Cyrtodactylus ND4 gene sequences was used to construct the phylogenetic tree. It was also added two outgroup sequences with a length of 611 bp. The validity of phylogenetic tree branches was confirmed based on the bootstrap values of Maximum Likelihood Bootstrap (MLBS), Maximum Parsimony Bootstrap (MPBS) and Bayesian Posterior Probability (BPP). The Cyrtodactylus species were grouped based on its genetic similarities. Phylogenetic tree of Cyrtodactylus showed that Cyrtodactylus was a monophyletic group which divided into two major clades (B and C). Clade A has a single species that is different from the other Clades (Figure 1).

![Figure 1. Phylogeny Tree of Cytodactylus based on the three different consensuses ML, MP and BI. (*indicated more than 70 %)](image)

The consensus of Cyrtodactylus phylogenetic trees showed a monophyletic relationship and a grouping based on the geographical boundaries. Clade A was a Cyrtodactylus group originating from West Java. However, there was a separation of clade B which is Cyrtodactylus group originating from Central Java. The clade C was containing a group of Cyrtodactylus which were originating from Sumatra, Kalimantan and Papua. The position of *C. marmoratus* taxon from West Java showed an interesting result. It can be clearly seen that *C. marmoratus* from West Java experienced a clade separation from *C. marmoratus* from Central Java. These relationships are well-supported (95/99/93) and the population of West Java has an uncorrected p-distance difference of 33% from *C. marmoratus* and 26% from *C. semiadii*. Besides, the taxon position of *C. marmoratus* was a sister lineage of all Cyrtodactylus populations in Java and Sumatra.

The Clade A and B belonged to different groups in the regions of Central Java and West Java although both clades were derived from Java island. Interestingly, the groups of Cyrtodactylus originated from Java were found separating. *C. marmoratus* from West Java was separated from the clade of *C. semiadiii* and *C. marmoratus* from Central Java. This result is which stated that *C. marmoratus* from Java is grouped with *C. marmoratus* from the Wallacea region [9]. It means that *C. marmoratus* is a species complex with an unclear type of locality [7].

In this study, the morphological characterization was used to confirm the *C. marmoratus* which grouped as one based on the results of the phylogenetic tree. The characterization was done using a single male specimen. It was characterized that *C. marmoratus* from West Java has tubercles on a
ventrolateral fold. The morphological characters that showed differences were no pore on precloacal scales, no continuous precloacal femoral pore, not deep precloacal groove, 37 ventral scales and 20 lamellae sub digital on the fourth finger. The result of this characterization was different from [10], which stated that *C. marmoratus* does not have tubercles in the ventrolateral folds, the amount of continuous precloacal pores is 45-50, the number of lamellae sub-digital on the fourth finger is 20-24. Thus, the *C. marmoratus* used in this study should be a new species.

There were two clades of *Cyrtodactylus* on phylogenetic tree originated from Java. The *C. marmoratus* originated from Sukabumi, West Java was collected on the mountain valley which allows this species to be isolated. The species isolation led to the adaptation towards new environments, so the morphological differentiation due to altered gene expression can be influenced [11]. The mountain range in the centre of West Java started from the center to the southern part of the Java island was formed valleys that did not have connecting lines. This allowed the formation of a geographic barrier that separates clades A and B which are still in the same island with adjacent crossings. Java is divided into small populations due to isolated mountain barriers without a connector [12]. This condition allows the breakdown of gene flow between populations from the west and Central Java.

The *C. marmoratus* originated from Nusakambangan, Central Java was grouped with *C. semiadii* from Cilacap. But it has been reported that the *C. semiadii* was different from *C. marmoratus* based on the morphological character, especially its short tail which has the same length as the SVL [4]. Although it’s different morphologically, its phylogenetic tree was closely related. The uncorrected p-distance value for both species was 28 %. In general, the *C. marmoratus* from West Java will form a clade with *C. marmoratus* from Central Java. But this result has strengthened the statement about the locality type of a *C. marmoratus* species complex in Java which is still not clear.

4. Conclusion
In conclusion, the geographical barriers caused by geological history led to the speciation of *Cyrtodactylus*. The *C. marmoratus* is a species complex due to its unclear locality type and distribution limits. In addition, about Java that became a small island due to the existence of mountain barriers which broke the genes flow. The further study using more samples, sensitive genes or more than one gene is still needed. It is also necessary to add samples originated from East Java to further classify the relationship of *C. marmoratus*

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