The nuchal gland system in Indonesian snakes: insights from function, structure, and evolution

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Introduction

The nuchal glands are organs of chemical defense embedded under the skin of several species of *Rhabdophis* and are known to contain toxins known as bufadienolides. The nuchal gland system is integrated by toxin sequestration, specialized morphology, and specific behavior. Snakes with the nuchal glands can consume toxic prey such as toads, sequester their bufadienolides, and further utilize them to deter predators. Despite Indonesia's unique environmental characteristics and its high level of biodiversity, the nuchal gland diversity of natricine species in this region has been rarely investigated. The nuchal glands have been already discovered in several *Rhabdophis* species of Indonesia, but there is no other detailed information, particularly regarding the organ's structure, function, and evolution. Therefore, I intend to investigate the nuchal gland system of snakes in Indonesia from the aspects of antipredator behavior, chemical prey preference, structures, chemical composition, and evolutionary history.

Materials and methods

I conducted the antipredator response experiment using artificial stimuli toward forty-eight individuals of *R. subminiatus* to investigate their antipredator behaviors, especially nuchal glands-related behavior. Eight behavioral responses were recorded, and the correlation of sex, body size, and body condition with these behaviors were analyzed. Chemical response test was conducted on *R. subminiatus* to find out its prey selection and the possible source of the toxins. Snakes were presented with prey chemical stimuli and their tongue flick frequency was analyzed. Gland morphology, including their features and arrangements, was examined in specimens with a trace of unusual structure in the interior side of the neck skin. Gland fluid of snakes and toads was extracted and analyzed to identify the chemical composition and to detect the presence of bufadienolides as confirmation of the presence of nuchal glands for snakes. Finally, three genes fragments of several natricine snakes of Indonesia were extracted, amplified, and sequenced. These sequences were used in the phylogenetic inference using Maximum Likelihood analysis and Bayesian Inference analysis.

Results and Discussion

All snakes performed body flatten and neck flatten, which suggests that these responses are their major antipredator behaviors. However, the tendency to perform the nuchal gland-related behavior declined as the body size increased. Furthermore, a negative correlation between the frequency of neck butting and body condition suggests that smaller snakes, particularly those below average body condition, depend more on the nuchal glands to deter predators. Chemical prey preference test suggested that *R. subminiatus* discriminates different potential prey even though snakes had been in captive for a relatively long period and their feeding was restricted to only frogs. The result also suggested that they would consume toads as their toxin source. Among several species of toads, snakes showed high number of tongue flicks toward *Ingerophrynus biporcatus* and *Duttaphrynus melanostictus,* and low number of tongue flicks toward *Phrynoidis aspera*. The morphological analysis confirmed that three species of natricine snakes have three different types of nuchal glands, particularly *Rhabdophis* species of Sulawesi, which possibly has a novel form. LC/MS analyses confirmed that snakes accumulate bufadienolides. Approximately 21, 44, and 26 bufadienolides detected from *R. subminiatus*, *R. flaviceps*, and *R. chrysargoides*, respectively. It is presumed that snakes chemically convert dietary bufadienolides from toads but also reutilize some bufadienolides readily. Bufadienolides profiles of snakes and toads indicated that their toxin composition is highly diverse. The specific bufadienolides identified in particular snake and toad species may provide evidence of independent evolutionary origins of certain bufadienolides modifications. The Maximum likelihood and Bayesian Inference trees resulted in almost identical topology. The analyses supported the highly divergent lineages of Indonesian *Rhabdophis* species, and substantial genetic divergence was observed within *R. flaviceps*, *R. chrysargos*, *R. chrysargoides*, and *R. callistus*. This significant genetic divergence may indicate that *Rhabdophis* species of Indonesia contain several undescribed species and/or possibly cryptic species. *Rhabdophis* species of Sulawesi was distantly separated from the other conspecifics particularly those within the nuchal gland clade. The phylogenetic position of *R. chrysargoides* and *R. callistus*, along with the discovery of their glands, provided a new indication of the multiple independent origins of the nuchal glands. Based on both phylogenetic analyses, it is strongly inferred that the common ancestor of *Rhabdophis* with the nuchal glands in Indonesia possessed no nuchal glands.

Conclusion

Overall, the present study contributes to the new findings on the functions, structures, and evolutionary history of the nuchal gland system within Indonesian snakes. In addition, the findings also documented the novel form of nuchal glands in the *Rhabdophis* species of Sulawesi. To conclude, this study showed that (1) *R. subminiatus* has nuchal glands-related behaviors to deter predators, which are affected by body size and conditions, (2) *R. subminiatus* shows high preference toward toads, which suggests that this snake would consume them to sequester the toxin, (3) the morphological structure and toxin components of the nuchal glands are diverse, and at least three types of nuchal glands are present in Indonesian snakes, and (4) the nuchal glands of Indonesian snakes have been independently evolved.