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Research Article
Variation of Protein Band Pattern on the Skin Secretions of *Chalcorana chalconota* (Schlegel, 1837) Complex

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Abstract

Background and Objective: *Chalcorana chalconota* is a complex frog species in West Sumatra which has been revised and designated as *Chalcorana parvaccola* and *Chalcorana rufipes* based on several studies such as morphology and genetics. Other studies such as protein band patterns can be a marker to differentiate species. This research was done to determine and prove the variations of protein band patterns found in skin secretions of the *C. chalconota* species complex. **Materials and Methods:** Frog samples were collected in the Pasia Laweh area, Pesisir Selatan, West Sumatra. The standard length measurement of the frog was carried out to determine the voltage that will be applied to the frog using an electric shock device (TAS/transcutaneous amphibian stimulator) in the process of removing the frog's skin secretions. Frog skin secretions were taken and used to see the pattern of protein bands using the SDS-PAGE method. **Results:** The protein band patterns of skin secretions of *C. chalconota* species complex were different between *C. parvaccola* and *C. rufipes*. In the skin secretions of *C. parvaccola*, there were eight protein bands with a molecular weight ranging between 12-103 kDa while for *C. rufipes* there were seven protein bands with a molecular weight ranging between 12-102 kDa. There were six protein bands shared by these two species. Two bands were only found in the skin secretions of *C. parvaccola* and one band was only found in *C. rufipes*. **Conclusion:** Pattern and molecular weight of protein in *C. parvaccola* and *C. rufipes* skin secretions can be used as protein markers to distinguish the two species.

Key words: Molecular weight, *C. chalconota*, *C. parvaccola*, *C. rufipes*, SDS-PAGE, frog skin secretion, protein band pattern

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Chalcorana chalconota is a complex species with a very varied morphology. *C. chalconota* has a slender body size, small to medium (adults 30-60 mm SVL) has a wide swimming membrane (webbing), the general colouration of *C. chalconota* is green on the dorsal side and white or cream on the ventral side. Genetic studies using the 16S rRNA gene have determined *C. chalconata* found in West Sumatra as *Chalcorana parvaccola* and *Chalcorana rufipes*. The morphological differences between *C. parvaccola* and *C. rufipes* lie in the size of the body, the colour of the swimming membrane, the size of the tympanum and the length of the third finger[1].

Several studies on the *C. chalconota* species complex in West Sumatra have been conducted by Inger *et al*.[1] regarding the Systematics of a widespread Southeast Asian frog, *Rana chalconota* (Amphibia: Anura: Ranidae), Yuliatmy *et al*.[2] regarding the genetic variation based on microsatellite DNA, Busta *et al*.[3] regarding the PCR-RFLP application for identification and authentication of *H. chalconota* species complex in West Sumatra. The results of these studies indicated that there were differences between *C. parvaccola* and *C. rufipes* based on morphology and DNA. However, there is no information regarding the pattern and molecular weight of protein from the skin secretions of *C. parvaccola* and *C. rufipes*.

Frog skin has some glands, including mucous and granular glands[4]. The secretion of mucosal glands causes the skin layer to become moist, which is necessary for the skin respiration and osmoregulation process[5]. The secretions of granular glands are used by frogs as a defence against predators because they contain bioactive molecules, antimicrobial peptides and toxic alkaloids[6]. Frog skin secretions also produce peptides, proteins, bio-amino, steroids and alkaloids which have different effects on predators[7]. Each frog species has peptides and proteins with different amino acid sequences[8]. Visualization through electrophoresis will show the protein band patterns which were caused by the separation of proteins based on the molecular weight.

Previous research on band pattern variations and molecular weight of Amphibian skin secretions was carried out by Daniel *et al*.[9] reported that the secretion of the parotoid gland of the toad *Rhinella marina* showed seven protein bands with molecular weights ranging from 6-195 kDa. Fusco *et al*.[10] reported that the secretion of the skin frog *Argenteohyla siemersi* showed six protein band patterns with molecular weights ranging from 15-55 kDa. Based on existing research on several Amphibian species, it can be estimated that there are variations in the protein band patterns on the skin secretions of *C. parvaccola* and *C. rufipes*, which are part of the *C. chalconota* species complex. This study aims to determine and prove the existence of variations in the protein band patterns on the skin secretions of *C. parvaccola* and *C. rufipes* which can be used as protein markers to distinguish the two species.

MATERIALS AND METHODS

Study area: This research was conducted from October, 2019 to January, 2020. The samples were collected in Pasia Laweh Village, Kambang, Pesisir Selatan, West Sumatra. Then the observations were continued at the Genetics and Biomolecular Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Padang.

Sample collection: The number of samples collected for this study was 46 individuals, where 29 individuals were *C. parvaccola* and 17 individuals were *C. rufipes*.

Standard length measurement (Snout-vent length/SVL): The standard length measurement (SVL) started at the tip of the frog's mouth and continued vertically until it reached the end of the tailbone. This SVL measurement needs to be done before the process of removing the frog's skin secretions to adjust the voltage that will be applied to the frogs[11].

Removal of frog skin secretions: The excretion of frog skin secretions was stimulated using an electric shock device (TAS/transcutaneous amphibian stimulator) based on Grant and Land[12] method with modified. A frog with a standard length/SVL of 21-40 mm was given a voltage of 4 volts, SVL between 41-60 mm was given 6 volts and an SVL greater than 60 mm was given 10 volts. Stimulation was carried out for 30 sec. The resulting frog skin secretions were put into a microtube and stored in a refrigerator.

Determination of the pattern of frog skin secretion protein bands: Determination of the protein band patterns of frog skin secretions was carried out using the SDS-PAGE method[13].

Determination of protein molecular weight (MW): SDS-PAGE photo data were used to qualitatively determine the molecular weight of the sample (protein of frog skin secretion) by comparing the bands produced by the sample with bands produced by the marker. The molecular weight of the sample

was determined quantitatively by measuring the migration distance of the protein (marker and sample) and dye (Bromophenol Blue). After that, the migration distance was calculated using the relative mobility (R_f) formula[14]:

$$\text{Formula relative mobility} = \frac{\text{Protein migration distance}}{\text{Dye migration distance}}$$

After the R_f marker and sample values were obtained, then it was continued with the determination of the log MW curve from the R_f marker and log MW marker values using the Microsoft Excel program. Next, the sample R_f value was used to determine the sample's MW based on the linear Eq.:

$$y = ax+b$$

The equation obtained from the log MW marker curve, x was the sample R_f[14]:

$y = ax+b$
$x = R_f$ unknown protein
$y = \log (MW)$

So, the inverse log (MW) is $MW = 10^y$.

RESULTS AND DISCUSSION

The research results of the protein band pattern of *C. chalconota* species complex skin secretions (*C. parvaccola* and *C. rufipes*) were shown in Fig. 1a-b and Table 1. The skin secretions of *C. parvaccola* contained eight protein bands and *C. rufipes* skin secretions had seven protein bands as shown in Fig. 1. There are six protein bands shared by the two frogs (*C. parvaccola* and *C. rufipes*), while the other bands showed different protein band patterns between the two species.

The difference in the number of protein bands between the two frog species showed that the protein banding pattern of frog skin secretions can be used as one of the distinguishing characteristics of species. This was following previous studies

Table 1: Molecular weight/MW (kDa) of protein from *C. chalconota* species complex skin secretions

Chalcorana parvaccola	MW (kDa)	*Chalcorana rufipes*	MW (kDa)
Band 1	103	Band 1	102
Band 2	83	Band 2	83
Band 3	66	Band 3	66
Band 4	46	Band 4	56
Band 5	36	Band 5	46
Band 6	30	Band 6	31
Band 7	24	Band 7	12
Band 8	12		

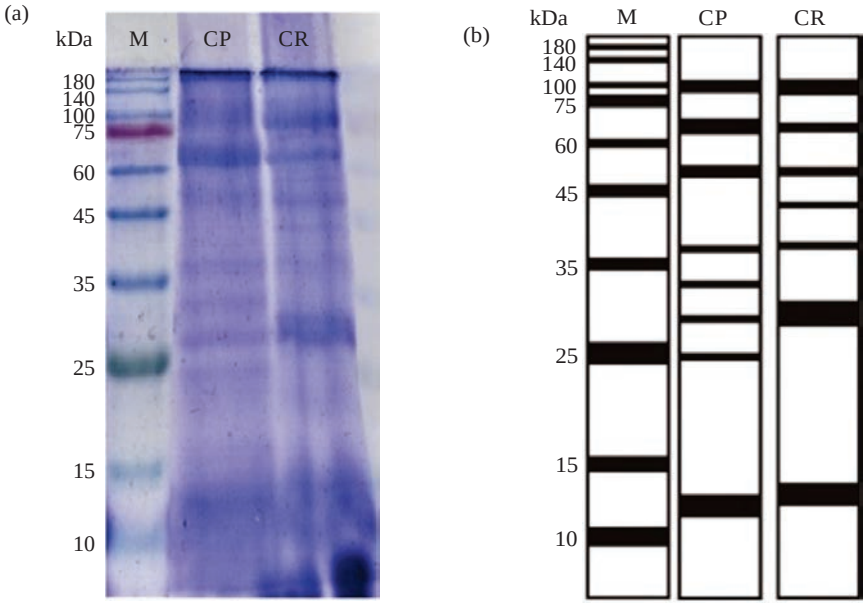

Fig. 1(a-b): (a) Electrophoresis results on polyacrylamide gel electrophoresis (SDS-PAGE) of frog skin secretions *C. parvaccola* and *C. rufipes* and Marker (b) Visualization of SDS-PAGE results from *C. parvaccola* and *C. rufipes* skin secretions using ImageJ and paint application
kDa: Unit weight of protein, M: Marker, CP: *C. parvaccola* and CR: *C. rufipes*

reported by Nikolaieva *et al*.[15] that toad skin secretions in two species of the genus Bombina, namely *B. bombina* there were five protein bands and on *Bombina variegata* was obtained seven protein bands. The five protein bands found in *B. bombina* were also found in *B. variegata*, while the other two bands were only found in *B. variegata*. In the skin secretions of two *Bufo* species, namely *Bufo bufo*, five protein bands were obtained and on *B. viridis* was obtained nine protein bands. In the skin secretions of two *Pelophylax* species, which were *Pelophylax ridibundus* eight protein bands were obtained and on *Pelophylax esculentus* six protein bands were obtained. Other studies have also shown that the number and variation of protein bands for each Amphibian species were different. The parotoid gland secretion of the *R. marina* toad has seven protein bands[9]. *Argenteohyla siemersi* frog skin secretions had six protein bands[10].

The differences in the number of protein bands and molecular weight in the skin secretions of *C. parvaccola* and *C. rufipes* were shown in (Table 1). Proteins from *C. parvaccola* skin secretion have a molecular weight ranging from 12-103 kDa, while *C. rufipes* proteins from skin secretion have molecular weights ranging from 12-102 kDa. Table 1 showed that in the protein bands of the *C. parvaccola* and *C. rufipes* skin secretions there were six bands with the same molecular weight, also there were two bands that are only found in the skin secretions of *C. parvaccola* with a molecular weight (MW) of 36 and 24 kDa and one band were only found in *C. rufipes* with MW of 56 kDa.

These results indicated that protein bands with MW 36 and 24 kDa can be used as protein markers for *C. parvaccola* species and protein bands with MW 56 kDa for *C. rufipes*. This was following previous studies which reported that toad skin secretions in two species of the genus *Bombina*, namely *B. bombina* have protein bands with molecular weights ranging from 7-42 kDa and *B. variegata* has protein bands with molecular weights ranging from 7-102 kDa. Skin secretions of *B. bombina* and *B. variegata* have five protein bands with the same molecular weight, while the other two are only found in the skin secretions of *B. variegata*. For two species of the genus *Bufo*, *B. bufo* has protein bands with molecular weights ranging from 29-72 kDa, while *B. viridis* has protein bands with molecular weights ranging from 8-68 kDa. Two species of the genus *Phelophylax* namely *P. ridibundus* have protein bands with molecular weights ranging from 14-149 kDa and *P. esculentus* has protein bands with molecular weights ranging from 11-115 kDa[15]. The parotoid gland secretion of *R. marina* toads has protein bands with molecular weights ranging from 6-195 kDa. *Argenteohyla siemersi* frogs have protein bands with molecular weights ranging from 15-55 kDa[10].

Inger *et al*.[1] have revised and described the *C. chalconota* species complex in West Sumatra into *C. rufipes* and *C. parvaccola* based on the 16S rRNA gene and morphological characters. Busta *et al*.[3] reported the results of the analysis of 16S rRNA gene sequences and PCR product restriction using the Ava II restriction enzyme based on the PCR-RFLP method which was able to differentiate between *C. parvaccola* and *C. rufipes*. The results of this study also supported previous studies that were able to differentiate between *C. parvaccola* and *C. rufipes* frog species.

CONCLUSION

The results showed that there were variations in the band patterns and molecular weight of the protein from skin secretions of *C. parvaccola* and *C. rufipes* which could be used as protein markers to distinguish the two species. Protein bands with molecular weights of MW 36 and 24 kDa were markers for *C. parvaccola* species while MW 56 kDa were markers for *C. rufipes.*

SIGNIFICANCE STATEMENT

This research was conducted to determine the differences in *Chalcorana chalconata* complex at the protein level and is expected to help add scientific data to molecular biology, especially in terms of the classification of *C. parvaccola* and *C. rufipes* species which are part of the *C. chalconota* complex frog based on the protein banding pattern in the frog skin secretions.

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