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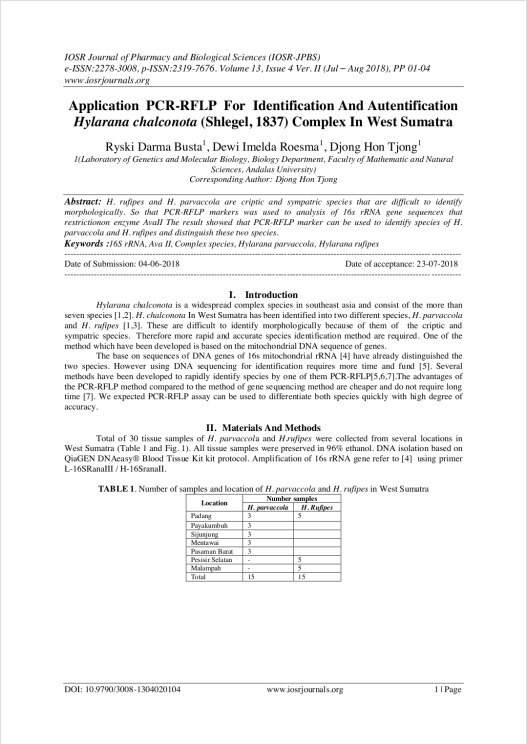

IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 13, Issue 4 Ver. II (Jul – Aug 2018), PP 01-04
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Application PCR-RFLP For Identification And Autentification *Hylarana chalconota* (Shlegel, 1837) Complex In West Sumatra

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1(Laboratory of Genetics and Molecular Biology, Biology Department, Faculty of Mathematic and Natural Sciences, Andalas University)
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Abstract: *H. rufipes and H. parvaccola are criptic and sympatric species that are difficult to identify morphologically, So that PCR-RFLP markers was used to analysis of 16s rRNA gene sequences that restrictionon enzyme AvaII The result showed that PCR-RFLP marker can be used to identify species of H. parvaccola and H. rufipes and distinguish these two species.*

Keywords *:16S rRNA, Ava II, Complex species, Hylarana parvaccola, Hylarana rufipes*

Date of Submission: 04-06-2018 Date of acceptance: 23-07-2018

I. Introduction

Hylarana chalconota is a widespread complex species in southeast asia and consist of the more than seven species [1,2]. *H. chalconota* In West Sumatra has been identified into two different species, *H. parvaccola* and *H. rufipes* [1,3]. These are difficult to identify morphologically because of them of the criptic and sympatric species. Therefore more rapid and accurate species identification method are required. One of the method which have been developed is based on the mitochondrial DNA sequence of genes.

The base on sequences of DNA genes of 16s mitochondrial rRNA [4] have already distinguished the two species. However using DNA sequencing for identification requires more time and fund [5]. Several methods have been developed to rapidly identify species by one of them PCR-RFLP[5,6,7].The advantages of the PCR-RFLP method compared to the method of gene sequencing method are cheaper and do not require long time [7]. We expected PCR-RFLP assay can be used to differentiate both species quickly with high degree of accuracy.

II. Materials And Methods

Total of 30 tissue samples of *H. parvaccola* and *H.rufipes* were collected from several locations in West Sumatra (Table 1 and Fig. 1). All tissue samples were preserved in 96% ethanol. DNA isolation based on QiaGEN DNAeasy® Blood Tissue Kit kit protocol. Amplification of 16s rRNA gene refer to [4] using primer L-16SRanaIII / H-16SranaII.

TABLE 1. Number of samples and location of *H. parvaccola* and *H. rufipes* in West Sumatra

Location	Number samples	
	H. parvaccola	*H. Rufipes*
Padang	3	5
Payakumbuh	3	
Sijunjung	3	
Mentawai	3	
Pasaman Barat	3	
Pesisir Selatan	-	5
Malampah	-	5
Total	15	15

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by Dewi Imelda Roesma

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Submission ID: 1271018343
File name: OSR_-_Ryski_Darma_Busta,_Dewi_Imelda_Roesma,_Djong_Hon_Tjong.pdf (369.76K)
Word count: 1966
Character count: 10813

IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
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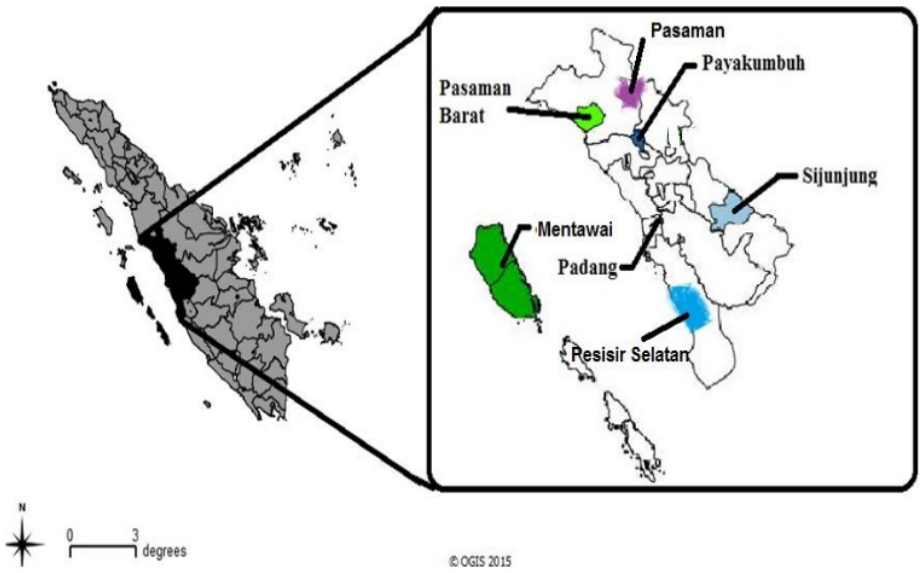

FIGURE 1. Sampling Location map in West Sumatra

Analysis of restriction enzyme site using *H.parvaccola* 16S rRNA gene sequence with accecing number DQ650394.1, DQ650395.1, DQ650399.1, EF487451.1, EF487453.1, EF487455 and *H. rufipes* accessing number DQ650396.1, DQ650397.1, DQ650398.1, EF487447.1, EF487448.1, EF487449.1. Analysis of the in-silico DNA-cutting reaction using the Cleaver program [8]

Digestion using enzyme *Ava* II is separately performed. PCR product amount 8 μl was mixed with 17 μl volume of reaction solution including 2.5 μl of 10 × M buffer and 0.5 μl of enzyme *Ava* II (New England). The mixture was incubated for 1 h at 370C for restriction reaction, and then for 10 min at 80 C for deactivation.

The digested PCR product with the restriction enzymes arranging side by side on 2% agarose gel for 30 min at 100 voltages and then stained with ethidium bromide. Finally, we validated specificity of the PCR-RFLP Pattern for each species by comparing position of the bands observed on the gel.

III. Results

We obtained a length of 735 bp sequences of the forward primer L-16s RanaIII and reverse H-16s RanaIII amplified between the 381-1116 bp of 1405 pb of the 16s rRNA gene *H.chalconota*. Based on this length gene we obtain *Ava II* (GGATA) restriction enzyme of the *H. parvaccola* and *H. Rufipes* on the three site 304-308 bp, 355-359 bp and 541-545 bp (counted from 5' tip of estimated priming site of forward L-16S RanaIII in AB530583). The species-specific restriction pattern are summarized in Table 2. For *H. runfixes*, we idenditified recognize site at 304-308 bp and 541-545 bp. The second restriction site is not recognizable because of polymorphisms in the 355-359 bp site to change the base order to GGWTA. For *H. Parvaccola* we identified restriction site at 355-359 bp and at 541-545 bp. The 304-308 bp site not recognize because of the mutation on nucleotide 196 bp.

TABLE 2. Summary of species-specific restriction site

Species	*Ava II*			Fragment Length
	Site I	Site II	Site III	
H. parvaccola	GGAC<u>T</u>	+	+	360/195/188
H. rufipes	+	GGA<u>TA</u>	+	308/240/195

Underlined characters indicated nucleotide difference from recognized sequence

The electrophoresis result on *H. rufipes* samples showed the fragment of 16s rRNA gene after restricted by using *Ava* II enzyme has same pattern of then in-silico analysis. Restriction on two sites that mention above will obtained three bands (approx. 300 bp, 250 bp and 200 bp). All *H. rufipes* samples showed the same result (Fig 2).

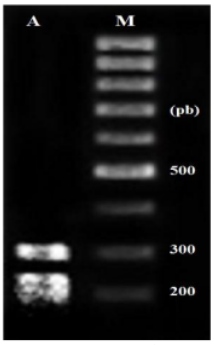

FIGURE 2. Bands of the Restriction enzyme *Ava* II on the sequences 16s rRNA gene *H. rufipes*

In-silico analysis on the *H. parvaccola* shows the restriction site on the 355-359 bp and 541-545 bp. However, on electrophoresis result we obtain three bands pattern for all of the samples. In Sijunjung show three band were obtained (approx: 500 bp, 350 bp and 200 bp) (fig.3A). 200 bp band probably consist of three bands were co migration. In this population shown that two allele, first recognize site 192-196 (approx: 500 bp and 200 bp) and another allele site 245-249 bp and 431-435 bp (approx. 350 bp, 200 and 200 bp). On Mentawai, Padang and Pasaman Barat populations we got two bands (approx. 500 bp and 200 bp (less clear band) which shows the two population have one recognize sitea 192-196 bp (fig. 3B, E and F). Therefore one sample form Padang and Payakumbuh population (fig. 3C and D) have two recognize site same with the in silico result (approx. 350 bp, 200 bp). The band 200 bp showed consis of two bands are co-migration.

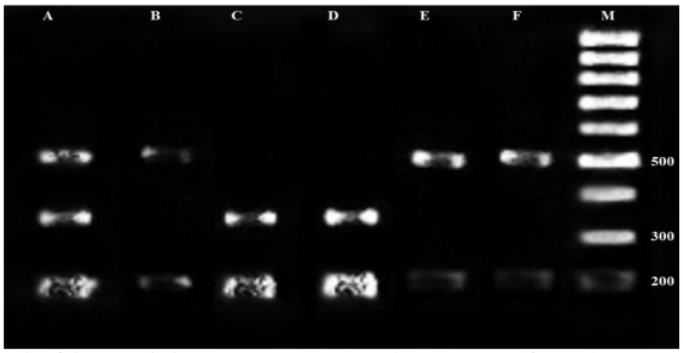

FIGURE 3. Bands of the Restriction enzyme *Ava* II on the sequences 16s rRNA gene *H. parvaccola*. A. Sijunjung B. Mentawai, C. Payakumbuh, D, E. Padang, F. Pasaman Barat

IV. Discussion

Analysis *Ava* II restriction enzyme on 735 bp amplification of 16s rRNA gene *H. rufipes* and *H. parvaccola* revealed both of them has two of three *Ava* II recognition site. We can distinguish these two species by recognize bands preview. *H. rufipes* has one band pattern and *H. parvaccola* has three band patterns after restriction by *Ava* II. *H. rufipes* has one band pattern because *H. rufipes* has identical 16S rRNA gene among individuals in West Sumatra. Previous research reported that individuals of *H. rufipes* had 0% sequence divergence[1].

The difference in the size and number restriction band on *H. parvaccola* is due to the high variation of 16S rRNA gene. The results of the study reported that *H. parvaccola* has a high divergence sequence between individuals in West Sumatra and *H. chalconota (H. parvaccol*a) from Mentawai is recommended to be a candidate species [1,9). *H. parvaccola* in West Sumatra also reported has high genetic variation based on DNA microsatellite [3].

Electrophoresis result on *H. parvaccola* from Padang and Payakumbuh populations showed identical with in-silico analysis. *H. parvaccola* from Padang, Mentawai and Pasaman Barat populations showed two bands (approx. 500 bp and 200 bp). *H. parvaccola* from Padang, Mentawai and Pasaman Barat populations. We assumed that population were from one common ancestor. Geographic history indicated Padang, Pasaman Barat and Mentawai area were connected through Batu Island [10, 11, 12].

H. parvaccola from Sijunjung population assumed to have two band patterns. This is because individuals of *H. parvaccola* from Sijunjung population have two types of mitochondrial called heteroplasmy. Heteroplasmy is the presence of more than one variant of mitochondrial DNA in cells or individuals[13]. Heteroplasmy case also found in Water Frogs (Pelophylax spp.) In Southern Europe[14].

This study showed that *H. parvaccola* and *H. rufipes* can be distinguished by using in-silico PCR-RFLP and also in-vitro PCR-RFLP assay. Therefore, we concluded that PCR-RFLP assay can used to indentify *H. parvaccola* and *H. rufipes* and differentiate both of them.

V. Conclusion

Based on sequences analysis of 16S rRNA and restriction PCR product by *Ava* II restriction enzyme revealed that PCR-RFLP assay can used to identifiy *H. parvaccola* and *H.rufipes* and differentiate both of them.

Acknowledgements

The authors would like to thank for Dean Of Mathematic and Natural Sciences Faculty, Andalas Univesity for giving funding for this research.

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