A STUDY ON THE GENETIC VARIABILITY OF STINGLESS BEES IN TERENGGANU BY USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS: A PRELIMINARY ASSESSMENT

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ABSTRACT

Heterotrigona itama and *Tetragonula fuscobalteata* are two common stingless bees species found in Malaysia. Information regarding the application of the molecular technique in genetic variation of the population of these species is very little. The aim of this study is to investigate the genetic variation of the *H. itama* and *T. fuscobalteata* by using Random Amplified Polymorphic DNA (RAPD) marker. Eight samples from each species were sampled from Merchang and Kuala Nerus, Terengganu. The extraction of DNA samples based on DNeasy Blood and Tissues Kit from Qiagen. The DNA purity and quality were measured by using BioDrop™µLITE dsDNA. The amplification of the DNA was conducted with OPA-12 and OPA-16 primers for *H. itama* and OPA-03 and OPA-09 primers for *T. fuscobalteata*. A total of 18 RAPD bands with 15 polymorphic bands (83%) ranging in size from 350-2500 bp were identified from *H. itama*. For *T. fuscobalteata*, 15 bands with 13 polymorphic bands (87%) with sizes ranging from 300-1250 bp. Firstly, the results indicated that the population of *H. itama* and *T. fuscobalteata* have high levels of polymorphism due to a low inbreeding factor within their population and the results also revealed that these species have genetic variability. The findings of this study will be used as basic genetic information for stingless bees' breeding programmes and conservation and management of these species in Malaysia.

Keywords: Genetic variation, *Heterotrigona itama*, *Tetragonula fuscobalteata*, RAPD, Stingless bee and DNA

ABSTRAK

Heterotrigona itama dan *Tetragonula fuscobalteata* merupakan dua spesies kelulut yang biasa dijumpai di Malaysia. Maklumat mengenai penggunaan teknik molekul dalam mengukur variasi genetik populasi spesies ini sangat kurang. Tujuan kajian ini dijalankan adalah untuk mengkaji variasi genetik *H. itama* dan *T. fuscobalteata* dengan menggunakan penanda *Random Amplified Polymorphic DNA* (RAPD). Lapan individu dari setiap spesies disampel dari Merchang dan Kuala Nerus, Terengganu. Pengekstrakan sampel DNA menggunakan *DNeasy Blood and Tissues Kit* dari Qiagen. Ketulenan dan kualiti DNA diukur dengan menggunakan BioDrop™

µLITE dsDNA. Amplifikasi DNA dilakukan dengan menggunakan pencetus OPA-12 dan OPA-16 untuk *H. itama*, manakala pencetus OPA-03 dan OPA-09 untuk *T. fuscobalteata*. Sebanyak 18 jalur RAPD dengan 15 jalur polimorfik (83%) dengan saiz jalur antara 350-2500 bp dikenal pasti dari *H. itama*. Untuk *T. fuscobalteata*, 15 jalur dengan 13 jalur polimorfik (87%) dengan saiz jalur antara 300-1250 bp. Hasil kajian menunjukkan bahawa populasi *H. itama* dan *T. fuscobalteata* mempunyai tahap polimorfisme yang tinggi kerana faktor pembiakan dalaman yang rendah dalam populasi mereka dan juga menunjukkan bahawa spesies ini mempunyai variasi genetik. Penemuan kajian ini akan berguna sebagai maklumat genetik asas untuk pembiakan, pemuliharaan dan pengurusan spesies ini di Malaysia.

Kata kunci: Kepelbagaian genetik, *Heterotrigona itama*, *Tetragonula fuscobalteata*, RAPD, Kelulut, DNA

INTRODUCTION

Stingless bees are classified under the order Hymenoptera, family Apidae and tribe Meliponini (Michener 2007; Wille 1983; Winston & Michener 1977). Stingless bees constitute a diverse group of highly eusocial insects that occur throughout tropical regions around the world (Ramírez et al. 2010). According to Rasmussen and Cameron (2010), at least 600 species of stingless bees were described in the world and classified under 60 genera. In Malaysia, there are approximately 32 to 35 species of stingless bees identified, locally known as 'kelulut' (Mohd Fahimee et al. 2016a; Mohd Norowi et al. 2010). Stingless bees are a common pollinator in Malaysia's agricultural ecosystems but no report has successfully proven them to be a major pollinator (Mohd Fahimee et al. 2016b; Wahizatul et al. 2019). Because of the high diversity of stingless bees, many species are still under study and more effort is needed to explore and learn about stingless bees.

Several molecular tools for the detection of genetic variation within species have been developed and Randomly Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) is one of the molecular genetic markers that has been widely used to study the genetic variability around the world (Williams et al. 1990). This technique was chosen as it is rapid, simple and beneficial. For instance, no prior knowledge of the genome is required and only a small quantity of DNA is needed to determine the genetic variability in many organisms (Hadrys et al. 1992). There have been many studies that have used RAPD technique to detect other species of stingless bees as reported by Tavares et al. (2001) who detected some polymorphic bands in *Melipona quadrifasciata* from Brazil. Waldschmidt et al. (2002) also found some polymorphic bands in *M. quadrifasciata* also from Brazil. Baitala et al. (2006) studied about RAPD polymorphism in *Tetragonisca angustula* (Hymenoptera; Meliponinae, Trigonini) populations which showed a high polymorphic degree in *T. angustula* in relation to values found for other stingless bees. RAPD marker was used to study the genetic variability of stingless bees in this study.

In this preliminary study, the genetic structure of *Heterotrigona itama* and *Tetragonula fuscobalteata* was investigated by assessing the genetic variability of these species. *Heterotrigona itama* is the most popular domesticated species and the most common species reared by local stingless beekeepers that have been well distributed throughout Peninsular Malaysia and Borneo (Abd Razak & Ishak 2017; Kelly et al. 2014). *Tetragonula fuscobalteata* is the smallest yet most antagonistic species of stingless bees with an average body length of 3.26 mm. This species can be domesticated, and is quite commonly found in stingless bee farms around Malaysia as well as the Universiti Malaysia Terengganu area. In Malaysia, few studies

have been conducted about the diversity of stingless bees (Mohd Fahimee et al. 2018; Salim et al. 2012) along with their foraging behaviour (Basari et al. 2018; Mohd Fahimee et al. 2018; Wahizatul et al. 2015), the pollination efficiency on several crops (Wahizatul et al. 2019) as well as the suitability of native plants used in landscape designed for stingless bee (Wan Mohamad et al. 2020). Studies have been done regarding the phylogenetic relationships of *H. itama* by using mitochondrial DNA (Cytochrome c oxidase I gene) that have been compared to the population structure of this species taken from16 samples from five localities in Peninsular Malaysia (Kedah, Kelantan, Melaka and Johor) (Mohd Yusof et al. 2018) but no samples have been taken from Terengganu as yet. Meanwhile, information regarding the genetic variability of stingless bees especially in Terengganu has not been available. Therefore, the objective of this study is to assess the genetic variability of *H. itama* and *T. fuscobalteata* in Terengganu by using RAPD-PCR technique. This study is important in providing a database about the genetic structure needed towards cultivation, conservation, and management of these species.

MATERIALS AND METHODS

Study Area and Sample Collection

Samples of *H. itama* were collected from Merchang, Terengganu (5.03203° N, 103.29389° E) and for *T. fuscobalteata* at Universiti Malaysia Terengganu (UMT) (5.41137º N, 103.08997º E) (Figure 1). Twenty individuals of each species were collected randomly from different colony. Samples were collected from their sites by using an insect net and then preserved in 95%. A total of eight individuals were showed positive results by using RAPD markers. Therefore, the study of genetic variability of *H. itama* and *T. fuscobalteata* only involved eight samples for each species.

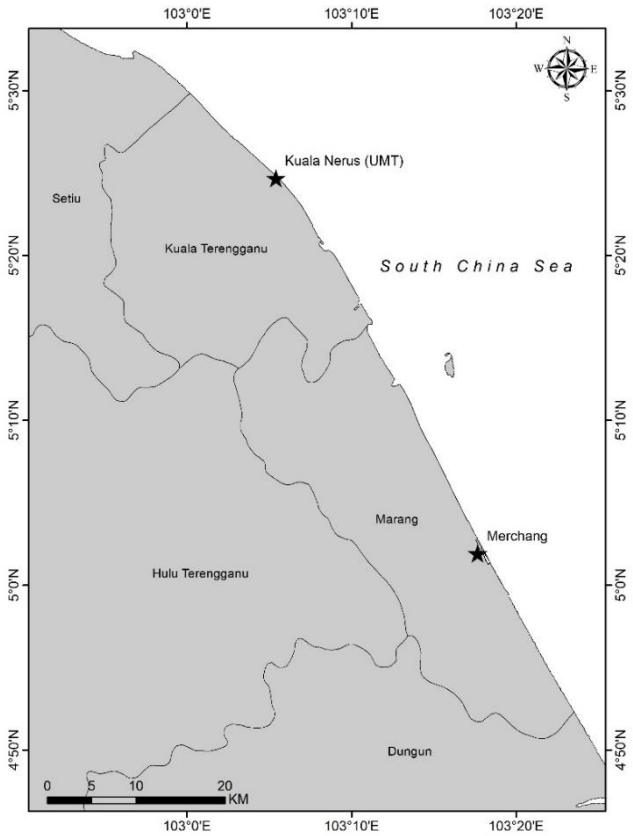

Figure 1. Location of the study areas

DNA Extraction and PCR Amplification

DNA was extracted from the whole body of the bee using DNeasy Blood and Tissue Kit (Qiagen's) protocol. The quantity DNA was measured by using BioDropTM µLITE ds DNA. Two RAPD primers were used, OPA-12 (5'-TCG GCG ATA G-3') and OPA-16 (5'-AGC CAG CGA A-3') for *H. itama* and OPA-03 (5'-AGTCAGCCAC-3') and OPA-09 (5'-GGGTAACGCC-3' for *T. fuscobalteata*. The amplification was carried out in a 25 µl reaction volume containing 1x reaction buffer, 50ng of genomic DNA, 1 mM of MgCl2, 1 mM of dNTPs. 2.5 mM primers and 2.5 U Taq DNA polymerase. Amplifying the DNA was carried out in a MJ PCR-200 thermal cycle (Bio- Rad)™ under the conditions: 45 cycles of denaturation at 95˚C for 30s, annealing process at 35˚C for 45s, 1 min for the extension of all primers at 72˚C and 7 min for final extension at 72˚C (Tavares et al., 2001; Tavares et al. 2007; Tavares et al. 2013). A negative control reaction (without DNA) was used in all PCR amplifications to identify possible contaminations. The PCR product was electrophoresed on 2% (w/v) agarose gel in 1x TBE buffer at 70 V for 1 h and 30 min. The gel was stained with SYBR Safe and photographed with Gel DocTM XR.

Data Analysis

The molecular weight of the bands was estimated based on the standard DNA banding patterns using 1 Kb ladder plus Vivantis, and 100 bp ladder (Promega) marker, and recorded in a binary matrix (0/1) to represent (1) as present or (0) as absent for a particular band for each individual. The bands were considered as polymorphic when they were absent in samples that had frequency greater than 1% (Jorde 1995). The genetic similarities between individuals were determined using Jaccard coefficients, and a dendrogram was constructed by applying the unweighted pair group method with arithmetic average (UPGMA) cluster analysis. Data were analyzed using the PAST software version 1.34 (Hammer et al. 2001).

RESULTS AND DISCUSSION

In this preliminary study, RAPD pattern of genomic DNA for all samples were generated by using two primers OPA 12 and OPA 16 for *H. itama*, and OPA 03 and OPA 09 for *T. fuscobalteata*. The total number of fragments, polymorphic fragments, proportion of polymorphisms and length of fragments of *H. itama* and *T. fuscobalteata*, is presented in Table 1 and Table 2, respectively. A total of 18 fragments were generated from *H. itama* and of those, 15 fragments were polymorphic. 15 fragments were generated from *T. fuscobalteata* and among the fragments 13 were polymorphic. There has been a lack of information on the genetic variability of stingless bees especially *H. itama* and *T. fuscobalteata* in Malaysia. This study showed that different primers generated the different RAPD profiles. All the primers used, generated amplification products of DNA with varying sizes, which are able to detect polymorphisms among the individual species. The fragment sizes ranging from 350 to 2500 bp were scored for *H. itama* and 300 to 1250 bp for *T. fuscobalteata*.

Table 1. Total number of fragments, polymorphic fragments, and proportion of polymorphism and length of fragment of *H. itama* for primer OPA-12 and OPA-16 for eight samples

Primer	Total number of fragments	Number of polymorphic fragments	Proportion of polymorphism (%)	Length of fragment (bp)
OPA -12	9	8	89	2500-350
OPA -16	9	7	78	2500-400
Total	18	15	83 (average)	

Table 2. Total number of fragments, polymorphic fragments, and proportion of polymorphism and length of fragment of *T. fuscobalteata* for primer OPA-03 and OPA-09 for eight samples

Primer	Total number of fragments	Number of polymorphic fragments	Proportion of polymorphism (%)	Length of fragment (bp)
OPA -03	7	6	86	1000-400
OPA -09	8	7	88	1250- 300
Total	15	13	87 (average)	

Banding patterns of RAPDs of *H. itama* using primer OPA-12 and OPA-16 that can be seen in Figures 2 and 3, respectively. Banding patterns of RAPDs of *T. fuscobalteata* are shown in Figure 4 and 5, respectively. The percentage of polymorphism for *H. itama* and *T. fuscobalteata* were 83% and 87%, respectively. The high polymorphism (genetic variability) revealed that the samples from *H. itama* and *T. fuscobateata* are genetically variable. The high level of *H. itama* and *T. fuscobalteata* indicates that inbreeding may not occur or occur at small rates. The existence of polymorphic markers indicated diversity among the populations at the genomic level (Lynch & Miligan et al. 1994; Williams et al. 1990). As a comparison, this study also referred to some studies on genetic variability of other species of stingless bees using RAPD that had been done in other countries. Tavares et al. (2001) studied the genetic variation of *M. quadrisfasciata* in Brazil and found that 25.23% polymorphism occurred in this stingless bee. Another study in Brazil by Waldschmidt et al. (2002) showed 44.6 % polymorphism in *M. quadrisfasciata*. Baitala et al. (2006) reported a high level of polymorphism in *T. angustula* with 87.72 %. In this study the similarity index for *H. itama* ranged from 0.38 to 0.91. Similarity index for *T. fuscobateata* ranged from 0.21 to 0.77. These similarity indices suggested that the genetic variability exists among individuals in each species. Generally, population with greater genetic variability have higher growth rates, developmental stability, viability, fecundity and resistance to environmental stress, and diseases (Dinesh et al. 1996). The high genetic variability suggested that the population of the species has a gene pool with sufficient genetic plasticity to support changes in the environment (Joaquim et al. 2009).

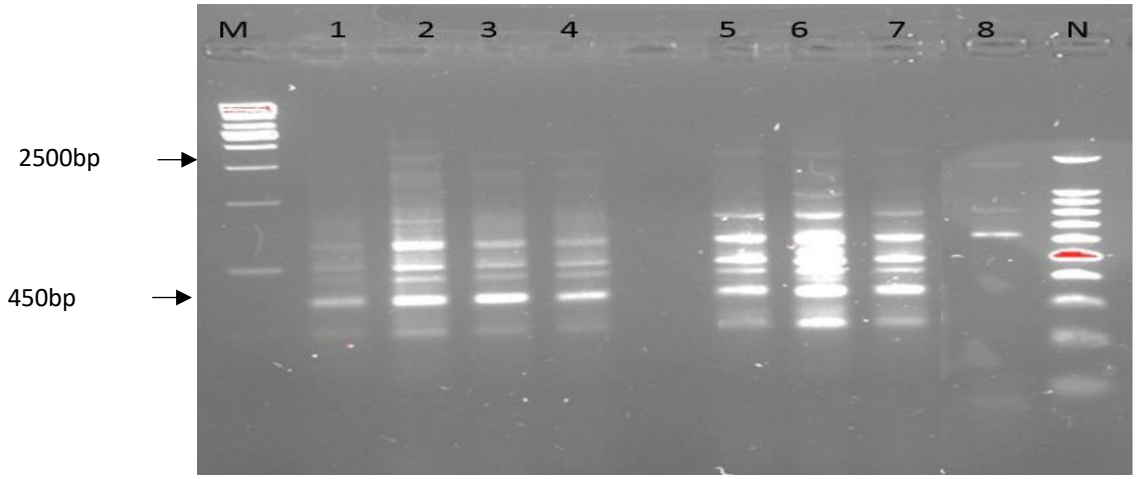

Figure 2. Banding patterns of RAPD fragments for *H. itama* using primer OPA-12. Lane M is marker 1 Kb DNA Ladder. Lane N is marker 100 bp DNA ladder. Samples 1-8 from Merchang

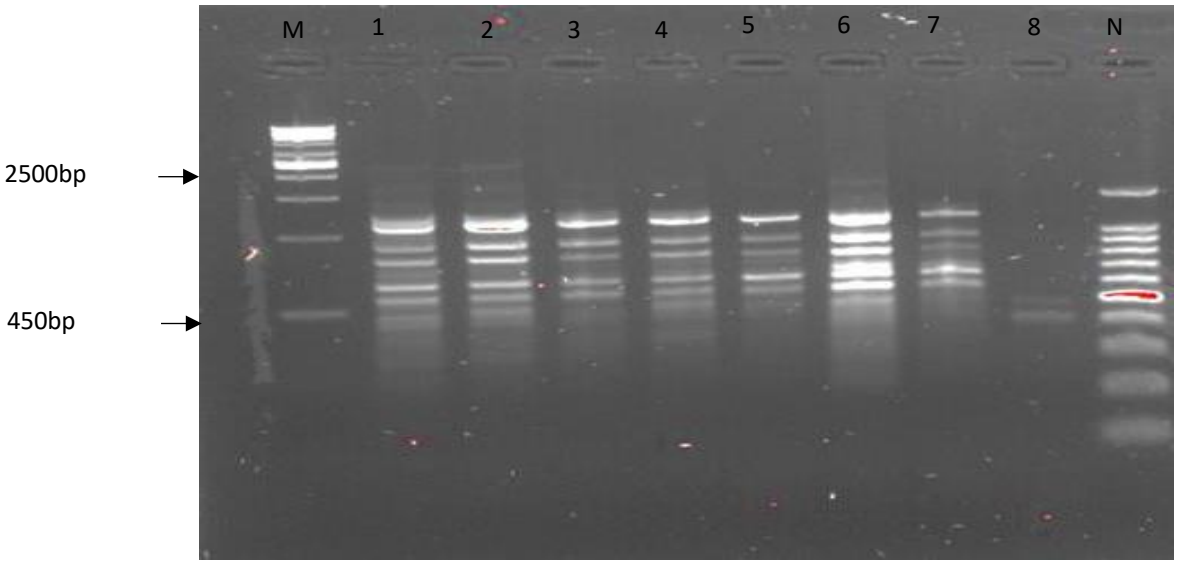

Figure 3. Banding patterns of RAPD fragments for *H. itama* using primer OPA-16. Lane M is marker 1 Kb DNA Ladder. Lane N is marker 100 bp DNA ladder. Samples 1-8 from Merchang

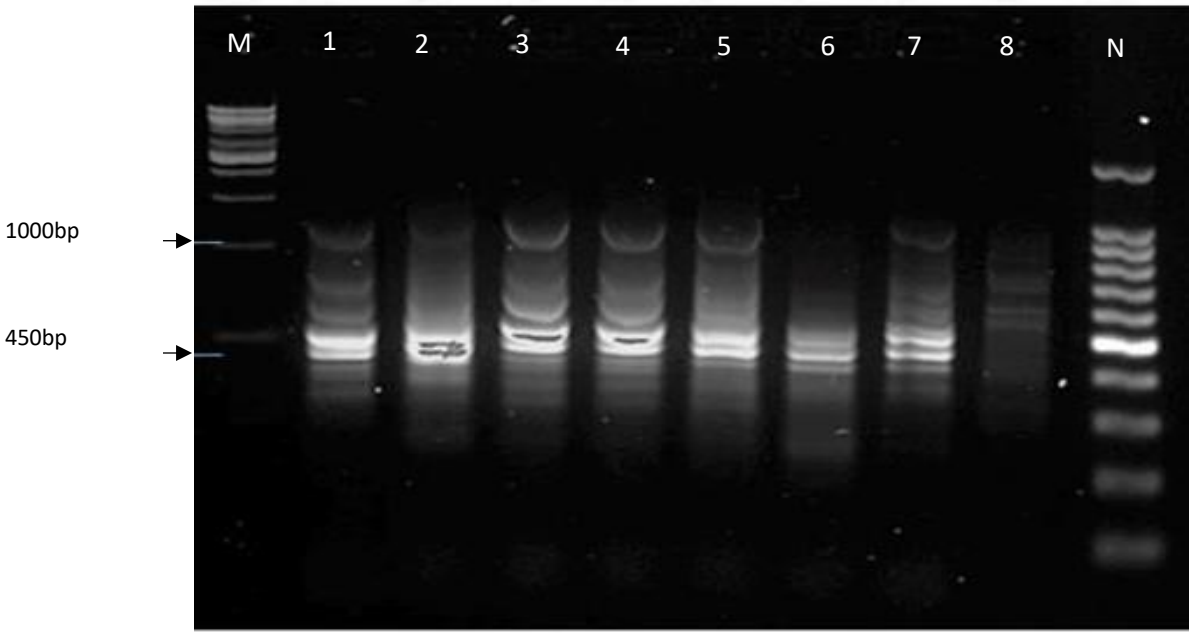

Figure 4. Banding patterns of RAPD fragments for *T. fuscobalteata* using primer OPA-03. Lane M is marker 1 Kb DNA Ladder. Lane N is marker 100 bp DNA Ladder. C is negative control. Samples 1-8 from Universiti Malaysia Terengganu

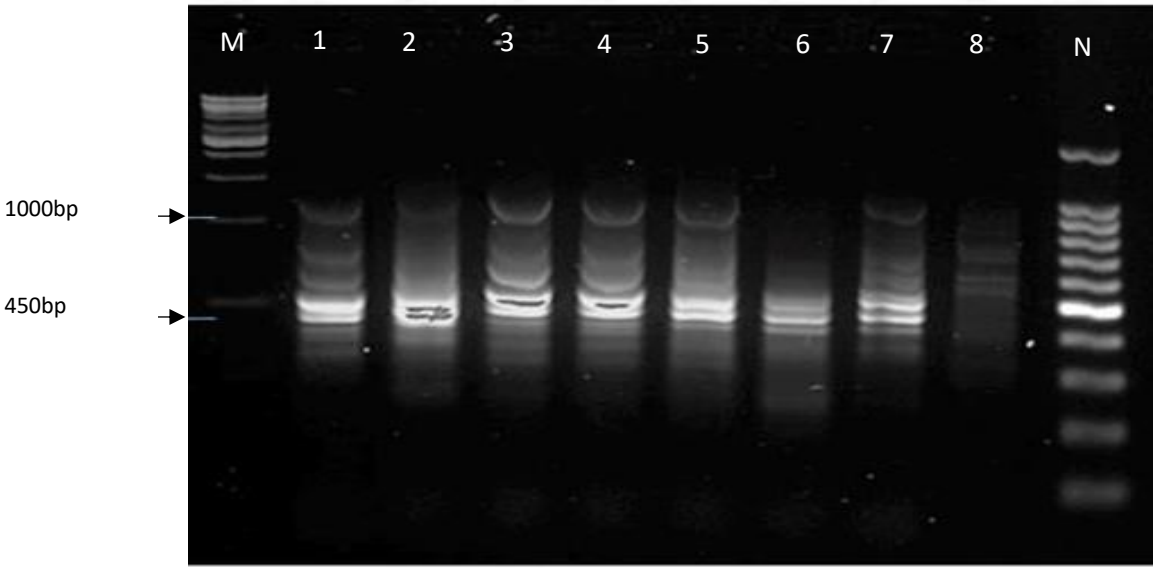

Figure 5. Banding patterns of RAPD fragments for *T. fuscobalteata* using primer OPA-09. Lane M is marker 1 Kb DNA Ladder. Lane N is marker 100 bp DNA Ladder. Samples 1-8 from Kuala Nerus (UMT)

CONCLUSION

RAPD markers successfully amplified the DNA of *H. itama* and *T. fuscobalteata* from Terengganu, even with limited sample numbers. This preliminary study result suggests that future studies must include more samples with more populations. Result also suggests the important for better understanding and maintenance of high levels of genetic variability of these species for brood stock selection and selective breeding programs especially for *H. itama* in the future.

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