Research projects of counterparts funded at UNJA in 2021

Name	Counterpart	Title
Revis Asra, Joko Ridho Witono, Izu Andry Fijridiyanto	**B14**	Sex identification of Jernang (*Daemonorops draco* (Willd.) Blume) using ISSR markers

Background and Objectives

Jernang (*Daemonorop draco*) as a synonym of *Calamus draco* (Baker 2015) is one of rattans which produces resin called dragoon's blood that has economic value as a medicine and natural dye (Gupta *et al*. 2007). This species belongs to the Palmae family, which is found in Sumatra and Kalimantan (Purwanto *et al*. 2005). In Sumatra, Jernang is distributed from West Sumatra, South Sumatra, Jambi, Riau to Lampung (Rustiami *et al*. 2004).

The resin is harvested from the fruit collected in the forest. The presence of *D. draco* in wild is rare (BKSDA Jambi 2010), and cultivation of this species is still rare (Asra and Farid 2016). Meanwhile, habitat destruction of *D. draco* for oil palm and rubber plantations is also causing a decline in the population of this species (Asra *et al*. 2014). Cultivation and conservation programs are needed to prevent the extinction of this plant in natural forest. Jernang is a dioecious plant. It is difficult to distinguish male and female plants based on vegetative characters. The sex of jernang can be determined by the structure of the inflorescence. Male plants produce inflorescences with fertile male flowers and female plants produce inflorescences with fertile female flowers and sterile male flowers. It takes 4–5 years for jernang to produce its first inflorescence (Asra *et al*. 2012).

In cultivation programs, it is desirable to grow as many female plants as possible to produce fruit containing dragoon's blood. In conservation programs, an equal (1:1) ratio of male and female plants is usually found in natural populations (Rottenberg 1998). It is essential to be able to identify male and female plants at the seedling stage. Therefore, it is important to find another method to distinguish male plants and female plants of jernang at the vegetative stage. The easiest and most reliable method for sex determination in plants is the use of sex-specific DNA markers. Such markers have been developed for many dioecious plants. In most cases, sex specificity was associated with the male sex (Aleksandrov *et al*. 2011). Among the molecular markers, inter-simple sequence repeat (ISSR) markers have been used to detect variations among plants and also to determine sex in various dioecious plants (Milewicz & Sawicki 2013; Grewal & Goyat 2015).

The Objective of this study is to develop genetic markers from ISSR analysis of male and female plants of *C. draco* to allow sex determination at seedling or vegetative stage in its life cycle.

Methods

Plant materials and Genomic DNA extraction

Leaf samples of 5 male, 5 female mature individuals and 10 seedlings of *D. draco* from Sepintun (Sarolangun Regency) in the Province of Jambi, Indonesia, were randomly collected and placed in silica gel prior to extraction. Total genomic DNA is extracted from silica gel-dried leaf tissues. DNA was isolated using Quick-DNATM plant/seed Mini-Prep Kit (Zymo Research) according to manufacturer manual. DNA samples of each plant was analysed individually to detect sex determination markers.

PCR (Polymerase Chain Reaction)

PCR amplification was performed in 16 µL reaction, and the reaction mixture contained 8 µL KOD One ™ PCR Master Mix-Blue (TOYOBO), 2 µL ISSR primer (10 pmol mL-1), 1 µL of DNA template and 5 µL of nuclease-free water. The DNA amplification was performed in a PCR instrument (TaKaRA PCR Themal Cycler Dice) for about 30 cycles. PCR procedures were performed in the following order: (1) one cycle of denaturation at 98 °C for 3 min: (2) 30 cycles of 98 °C for 10 s (denaturation), 60 °C for 3 s (annealing), 68 °C for 1 s (extension); and (3) final extension 68 °C for 7 minutes followed by soaking at 4 °C.

Electrophoresis

An initial screening revealed 26 ISSR primers from previous studies in some species of palms, of which 14 primers were successfully amplified (Table 1). PCR results generated by ISSR marker were processed by electrophoresis with 5 µL of the standard DNA, 100 bp DNA ladder of 1.5 % agarose gel in TBE 1X as the buffer solution. The agarose gel was then run on an electrophoresis device (Mupid ™ – exu Submarine) with 100 V for 90 minutes at room temperature. The resulting amplified bands were observed using gel documentation Gel Doc™ EZ Imager (BIO RAD). The band that was present in corresponding male or female samples and absent in the alternate sex samples was recognized as a potential sex-linked marker. The primers showing unique bands were further used with separate male and female seedling samples.

Results and Conclusion

Based on the PCR results using 26 ISSR primers, 14 primers of which were successfully amplified of 100-3000 bp with clear bands, whereas the other primers were not amplified or amplified with smear bands (Table 1). Some examples of successful PCR results are shown in figure 1.

Of all 14 primers tested and observed, only one primer, 844A [(CT)8AC] showed sex specificity in bulk analysis. The primer produced a unique 270 bp fragment (yellow arrow) in male bulk DNA, and this band was absent in female bulk DNA. Several other bands were also generated in both male and female samples in 340 bp and 800 bp (red arrow) (Fig. 2). Then the primer was presented to 5 seedling individuals (S6-S10) with similar banding patterns as in the male individuals (blue arrow). It could be concluded that seedling of S6-S10 are male individuals.

In natural stands, sexually mature plants are usually present, but flowering and fruiting occurs only between April and June. Based on this study, PCR-based DNA markers such as ISSR markers can be successfully used for sex identification at the seedling stage. However, designing and exploring other ISSR markers are necessary for gender determination of *C. draco* in the future.

As a conclusion, of all the 26 ISSR primers tested and observed, only one primer, namely 844A [(CT)8AC] showed sex specificity in bulk analysis. Designing and exploring other ISSR markers are necessary for gender determination of *C. draco* in the future.

Table 1. ISSR Primers were used in this study

No.	Primer Code	Oligonucleotide name	Sequence	Tm (0C)	Reference	PCR Products
1.	IS A02	(GA)9C	5'-GAGAGAGAGAGAGAGAGAC-3'	60	Al-Ameri *et al*. 2016	Amplified
2.	IS A71	(CA)8RG	5'-CACACACACACACACARG-3'	60	Al-Ameri *et al*. 2016	Amplified
3.	RT28	(CT)8G	5'-CTC TCT CTC TCT CTC TG-3'	60	Sarmah *et al*. 2017	Amplified
4.	RT29	(CA)8A	5'-CAC ACA CAC ACA CAC AA-3'	60	Sarmah *et al*. 2017	Amplified
5.	HB 9	(GT)6GG	5'-GTGTGTGTGTGTGG -3'	60	Younis *et al*. 2008	Amplified
6.	HB 11	(GT)6CC	5'- GTGTGTGTGTGTCC -3'	60	Younis *et al*. 2008	Amplified
7.	HB 12	(CAC)3GC	5'- CACCACCACGC -3'	60	Younis *et al*. 2008	Amplified
8.	814	(CT)8TG	5'- CTC TCT CTC TCT CTC TTG -3'	60	Younis *et al*. 2008	Amplified
9.	844A	(CT)8AC	5'- CTC TCT CTC TCT CTC TAC -3'	60	Younis *et al*. 2008	Amplified
10.	Clo 2	(CT)7YC	5'- CTCTCTCTCTCTCTCC -3'	60	Sarmah & Sarma 2011	Amplified
11.	ISSR2	(AAG)5 GC	5'- AAGAAGAAGAAGAAGGC -3'	60	Sarmah & Sarma 2011	Amplified
12.	UBC 807	(AG)8 T	5'-AGAGAGAGAGAGAGAGT-3'	60	Asra *et al*. 2014	Amplified
13.	UBC 808	(AG)8 C	5'- AGAGAGAGAGAGAGAGC-3'	60	Asra *et al*. 2014	Amplified
14.	UBC 834	(AG)8 YT	5'-AGAGAGAGAGAGAGAGYT-3'	60	Asra *et al*. 2014	Amplified

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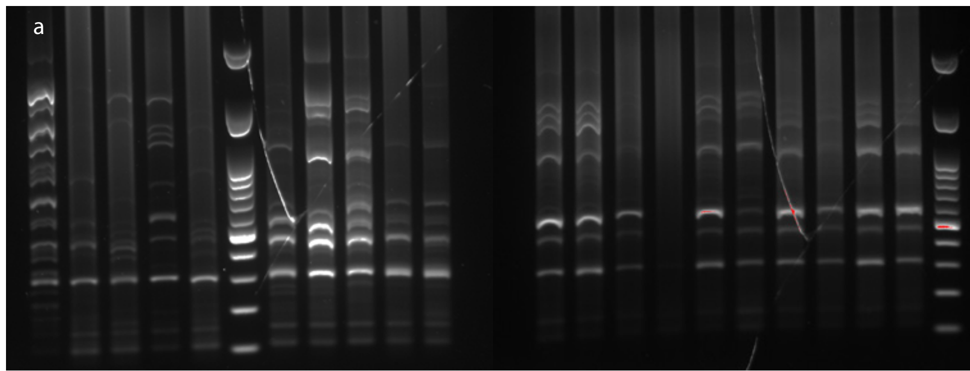

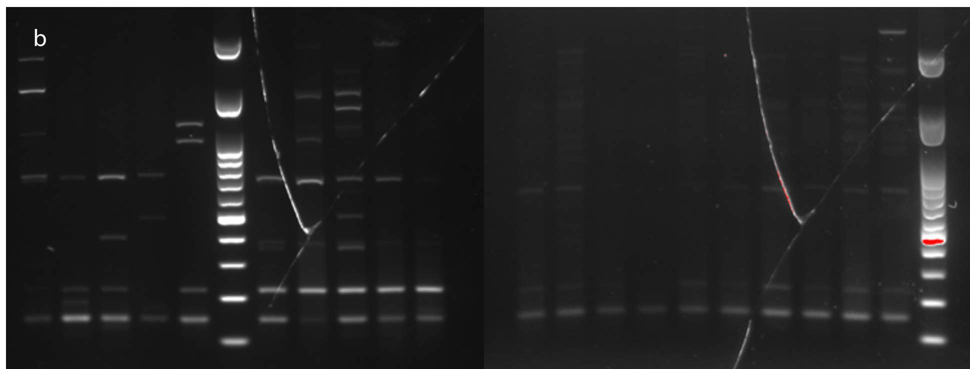

Figure 1. ISSR banding patterns of male, female and seedling (undetermined sex) individuals of *Calamus draco* obtained from 2 primers: (a) HB 9 and (b) HB 11. M = 100 bp ladder; F1-F5 = female individuals, M1-M5 = male individuals, S1-S10 = seedlings (undetermined sex).

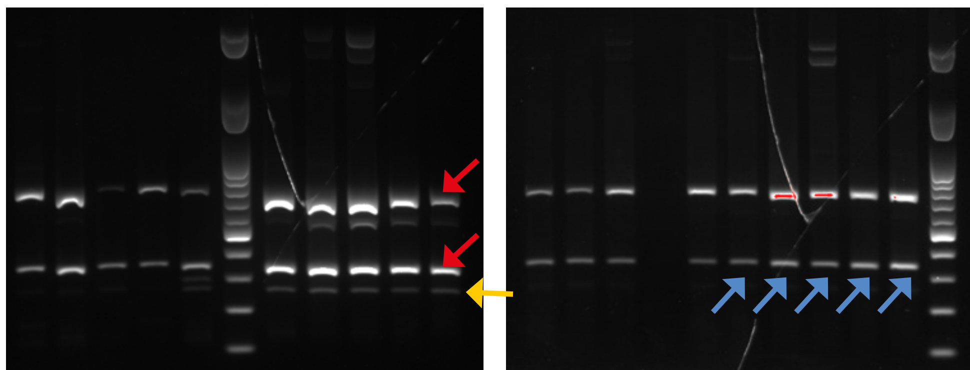

Figure 2. ISSR banding patterns of male, female and seedling (undetermined sex) individuals of *Calamus draco* produced by primer 844A [(CT)8AC]. M = 100 bp ladder; F1–F5 = female individuals, M1–M5 = male individuals, S1–S10 = seedlings (undetermined sex).

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