Revealing the Genetic Diversity of *Pinus merkusii* Aceh *via* Simple Sequence Repeat (SSR)

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Introduction

Around the globe, the genus Pinus is considered to be one of the most widely distributed genera of conifer trees, but only tropical pine (*Pinus merkusii)* grows naturally in the tropics, including on the Sumatran island. Three known native strains are known: the Aceh, Kerinci, & Tapanuli; with the first is known as the largest population among them. They grow naturally on the Seulawah Agam Mountain and in Middle Aceh (Figure 1d). Its resin is highly valued, classified as one of most valuable *Non-Timber Forest Products* (NTFPs), and it is worth up to US $ 50 million/year. The tropical pine forests have also played an immense role in maintaining the cool-climate condition of the tropical highlands, however, their existence over the past 2 decades have been threatened due to rapid deforestation, esp. the massive Citron grass (*Cymbopogon* sp.) plantation (Figure 1 a-c), that is highly valued as raw materials in the essential oil industries serving the wellness, spa-, & cosmetics' industries [1]. Moreover, approximately 1.5 m^2 logging Pinus wood is needed in order to run one single silo daily. Therefore, knowledge pertaining to their genetic diversity assessment is important in its conservation management [2].

Methodologies: DNA Analyses

The method is simplified on Figure 2a. Leaf samples were intended for the DNA analyses, in which they were collected from two different elevations on the Gayo highlands (n= 13 accessions): i) elevation < than 1, 000 m. above sea level (a.s.l.); ii) elevation > than 1.000 m. a.s.l. and these two areas in Aceh were applied as the major samples in this study.
Two additional regions, namely from Tapanuli in North Sumatera (n= 4 accessions), the Mount Kerinci (n=3 accessions) were also added. The samples were sent by post and these were served as 'outgroups'. Thus, the DNA of total 20 leaf samples of tropical pine were extracted based on Cetyltrimethylammonium Bromide (CTAB) (from SIGMA) modified from [3]. Seven SSR-markers [Forward (F); Reverse (R)] were applied (see: Table 1). The gradient Polymerase Chain Reaction (PCR) thermal cycler (Biorad) was using this condition: Temperatures (T) *vs*. annealing times: 98.8, 98.0, 68.0, 68.0 ^{0}C for 3.0 min., 10.0 sec., 1.0 sec., 7.0 min., respectively. Meanwhile, the Mupid-ex was applied for the gel electrophoreses (28 min.) and the visualization was performed by using GelDoc. Further calculation was calculated based on NTSYST.

Highlight & Conclusion

There are **still** three major tropical pine (P, merkusii) genetic diversity existing on the Sumatran island: (A) Aceh; (B) North Sumatera; (C) Kerinci Mountains in the Southern of Sumatra; with (A) is sub-divided into: (A.1) Seulawah strains and (B) Middle Aceh.
Our work was able to extract the DNA from the leaves. Out of 10 markers, only 7were able to be amplified. Thus, this study would enable us to quantify the amplitude of the tropical pine's genetic diversity and our result confirmed the large diversity based on Simple Sequencre Repeat (SSR) markers.

Methodologies: Morphological Analyses

In total, there were 15 morphological characteristics (*e.g.*: plant height, diameter of main stem, number of crown per branch, crown length, crown diameter, etc.) were measured manually by using measurement equipment *e.g.* measurement band, Compass, threads, etc. based on previous references. However, the results were already earlier published, elsewhere in 2021 [1] (Andini et al., 2021).

Result : Genetic Diversity Analyses

DNA Analyses

Prior to the main experiment, some trial and errors were made in order to meet the optimum PCR criteria. Each primer has different optimum temperatures, however, our results showed that the optimum temperatures of the gradient PCR were ranging from 45 up to 58.5 ^{o}C.
The DNA bands produced after electrophoretic analyses showed relatively very sharp bands (Figure 2b), although some primers (n=3 primers) were not succeeded to be amplified. Moreover, the bands resulted were varied or 'polymorphic'.
The dendogram analyses based on dissimilarity traits and produced four (N=4) major clades (Figure 2c), with a total of genetic distance was ranging from 0.00 to 0.88. The majority of the samples (≈80%) was put in clade 1. Interestingly, those samples from Tapanuli, namely Toba 1, Toba 2, Toba 4) and even those three accessions originated from the Mount Kerinci (Kerinci, 1,2,3) referring .s 'outgroup'. Cluster 2 had only two accessions: Blang Kejeren in Aceh Tengah & Toba 3. Meanwhile, the most genetic distance was found within the member of clade 1-6 and 1-4 with distances ranging from 0.305-0.32.

Figure 1 : a) the recently booming Citron grass plantation over the past two decades at high altitudes at the background: artificial *Pinus*' forest on Gayo highlands; b) horticultural crops (*e.g.* potatoes, red onions, or chili) have been planted massively on the costs of the natural and ancient *Pinus*' forests; c) a total of 20,852 ha Citron grass plantations are existing, in which approximately 93% are ready to be harvested, the concerning situation how the massive plantations of Citron grass have threatened the *Pinus*' forests. Moreover, Pinus trees are often logged-down for burning fuel for running the still traditional distillation process, that is majorly applied by many local farmers (PEMA, 2021); d) the natural and relatively still intact Pinus' forests in Kute Keramil near Ishak at elevation higher than 1,200 m. above sea level (a.s.l.)

Table 1: Seven Simple Sequence Repeat (SSR) primer information# applied in this *Pinus* study

No.	Primer ID	DNA Fragment Length (*bp*)	Repeated Motifs	Forward primer	Ta (°C)	Genebank ID
1	*pm01*	111-117	(TG)12	F: AGAGAAGGCACGATTTTGTC	56	AB201535
				R: TCCCACTAATCACTTTGAAAG		
2	*pm05*	112-118	(TG)9	F: GAGTCTAATTGCAAACCCCA	52	AB201537
				R: TGGAGATCTACCACTTTTTC		
3	*pm07*	284-309	$(AC)_8(AT)_4$	F: GAATCTAAGCATATGAAATGAG	52	AB201538
				R: CTTGTTAATGCTACTAGTTATG		
4	*pm09a*	81-99	$(AT)_5(GT)_{18}(AT)_2$	F: CCTTCTCATTTCGATATGCAC	52	AB201540
				R: ATTAAAGGTTATATGGGGCT		
5	*pm12*	181-193	$(GT)_5CT(GT)_5(AT)_5$	F: GAACAATCATTGCGGGTCCCG	59	AB201541
				R: ATGCTGCGTTTATATGTATAAGTGTC		
6	*Pde5*	116-235	$(CA)_5C(CA)_{51}$	F: AACGCACCTTTCTCAATGCAC	56	ABO38258
				R: ATAAAGAGGCTACATGGTCCC		
7	*SPAC 11.6*	165-200	$(CA)_{28}(TA)_7$	F: CTTCACAGGACTGATGTTCA	56	AJ223767
				R: TTACAGCGGTTGGTAAATG		
	Ta= Annealing Temperature					

this information is originated from Nurtjahjaningsih et al., (2007)

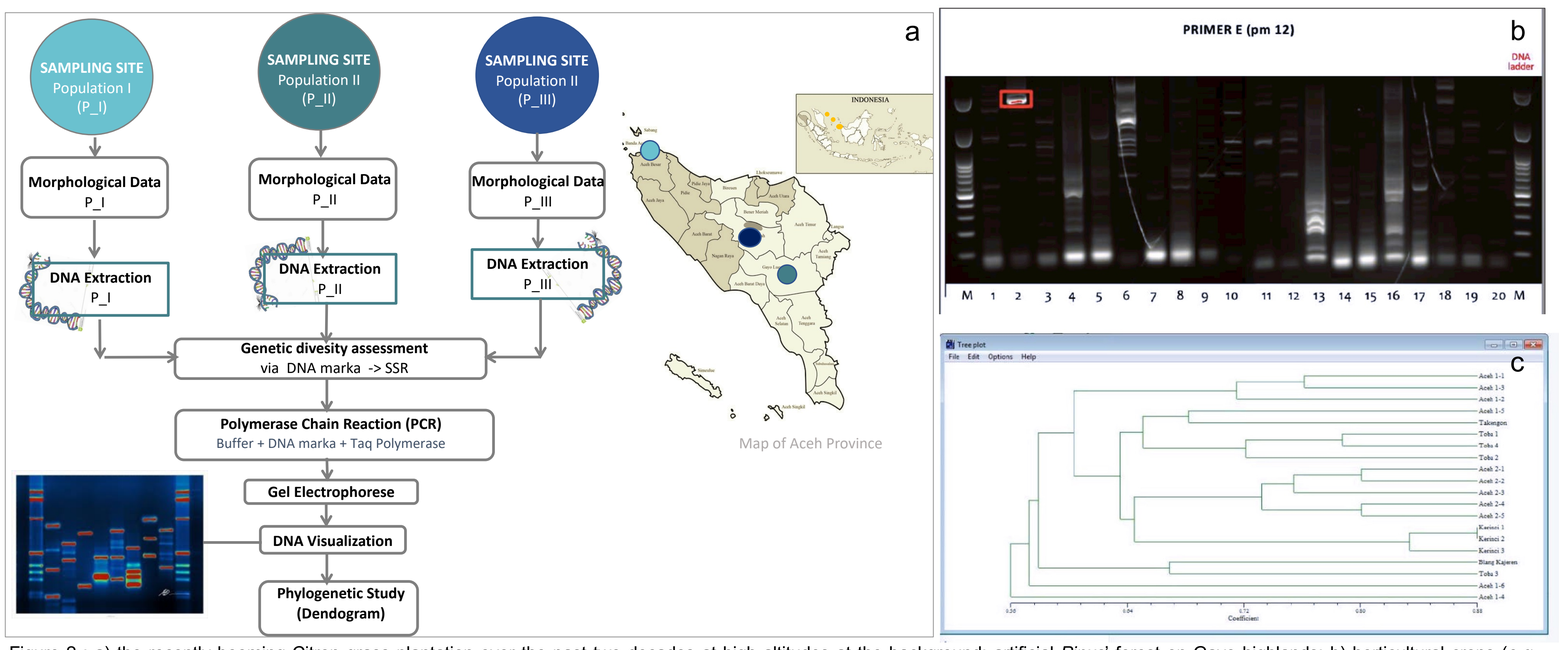

Figure 2 : a) the recently booming Citron grass plantation over the past two decades at high altitudes at the background: artificial *Pinus*' forest on Gayo highlands; b) horticultural crops (*e.g.* potatoes, red onions, or chili) have been planted massively on the costs of the natural and ancient *Pinus*' forests; c) a total of 20,852 ha Citron grass plantations are existing, in which approximately 93% are ready to be harvested, the concerning situation how the massive plantations of Citron grass have threatened the *Pinus*' forests. Moreover, Pinus trees are often logged-down for burning fuel for running the still traditional distillation process, that is majorly applied by many local farmers (PEMA, 2021); d) the natural and relatively still intact Pinus' forests in Kute Kerami situated at elevation higher than 1,200 m. above sea level (a.s.l.)

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