

The Analysis of *Pandanus* Relationship of Purwodadi Botanical Garden Collections Based on Morphological Character and Molecular Marker (*trnL* and *trnL-F*)

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ABSTRACT

The morphological characteristics of plant vegetative organ has the potential to be the defining factor of *pandanus* family collection at Purwodadi Botanical Garden (PBG). However, the information is not enough to confirm the identity of *pandanus* at the type-level. This problem happened at the collection of PBG. It is an urgent issue considering PBG as one of the most important ex-situ plant conservation institute that also has function as a study source for plant taxonomy science in Indonesia. Therefore, the use of molecular technology analysis using molecular marker (primer) is incredibly important to identify the member of *pandanus* family by identifying its family relationship pattern. This information was expected to give a clarity about the identity of *Pandanus* family at PBG which will support further research and activity for *pandanus* conservation. The plant sample includes 16 specimens of *pandanus* with one member of the family from *Freycinetia* as the outgroup. Morphological analysis of the 50 characteristics of vegetative organs (leaf, stem, and root) was conducted. Molecular analysis of DNA using primer of *trnL* and *trnL-F*. The data sequence as a result of the analysis was then tested using homology test with BLAST. All of the DNA sequence was paralleled using *Clustal W* analysis, the tree construction relationship was analysed using *Maximum Parsimony* (MP) method with

MEGA 6.0 program. The results of morphological studies showed the character vegetative that is the position of the spines on the leaves, the shape of apex leaf and the condition of the root surface above the soil. Molecularly, appears 1 new genera that has not been previously identified by PBG researcher is *Benstonea*.

Keyword: Morphology; *Pandanus*; Phylogenetic; Primer *trnL*; *trnL-F*.

INTRODUCTION

The *Pandanus* is a member of the *Pandanaceae* family which has a morphological character with the highest variation and the largest number of species compared to the *Freycinetia* (Buerki et al., 2012) and *Benstonea* (Callmander et al., 2012) in Indonesia. Generally, *pandanus* is morphologically easy to recognize because it has a distinctive character of prop-root, grows erect, not propagative, linear shape and rigid, tristichous spirals, have spines, no petioles; free or united Carpel, yellowish green to reddish yellow of fruit (drupe); Adaxial stigma, connate stamen (Backer & Bakhuizen, 1968; Callmander et al., 2012). In most members of the *Pandanaceae*, especially the *Pandanus* (*pandan*) of Purwodadi Botanical Garden (PBG) collections, its generative organs cannot always be encountered in a complete state at the same time (such as rarely stamen) (Stone, 1983; Callmander et al., 2012). The absence

of generative organs can also be caused by external factors such as habitat changes of environment as well as internal factors of N and P content in generative pandan organs (Minden & Kleyer, 2014). Incomplete generative organs can be represented by vegetative organs in overcoming difficulties in determining the position of the pandan taxon.

Determining taxon positions may be based on morphological character of vegetative organ as it has more easily visible and available character over time (Rahayu & Handayani, 2008), so it can be quickly and rapidly assessed. Although the vegetative organ is susceptible to change due to its high plasticity, it is important for certain plant species of taxonomy such as *Pandanus* (Hidayat et al., 2008). Research of Rachma et al., (2013) previously mentioned that character of vegetative organ is quite adequate as distinguishing feature among genera. Some of the vegetative organ characters becoming the key of distinguishing types are the presence and position of spines on pandan leaves, the apex shape, and the condition of the root surface above the soil. However, these characters are less able to confirm the identity of some pandan collection at the species level. This is a problem that occurs in PBG's pandan collection.

Difficulty in determining the position of pandanus taxon which is undergone by PBG needs to be solved immediately, considering PBG as one of the ex-situ plant conservation institutions, it becomes one of the learning sources of taxonomic plants in Indonesia (Soerohaldoko et al., 2001). Therefore, the use of molecular analysis technology becomes a very effective alternative way to obtain more comprehensive information about the supporting character of *Pandanus* identity through the pattern of kinship

relationship (Shukla & Misra, 1997). So far, research on phylogeny based on *Pandanus* DNA analysis has not been done much except by Callmänder (Callmänder & Laivao, 2003). In Indonesia, research on pandanus is still limited to genetic diversity (Panda et al., 2012; Wakte et al., 2012). This genetic information is expected to be used to confirm the taxon position of the pandan based on the vegetative organ that has been done before.

One of molecular markers commonly used by researchers to determine the phylogenetics of plant is by chloroplast DNA (*trnL* and *trnL-F*). Both primers are very effective in identifying members of the Pandanaceae (Callmänder et al., 2012; Callmänder et al., 2013; Gallaher et al., 2015). It is also supported by the advantages of two primers such as simple genomes because they have a medium-size genome length between 260-1000 bp, stable genetic structure, never or very rarely undergoing gen recombination, making it easy to amplify and to analyze (Dong et al., 2012; Hidayat et al., 2008). According to Buerki et al., (2012); Callmänder et al., (2012); Gallaher et al., (2015); Santos et al., (2011), several members of the *Pandanus* have been analyzed for their phylogenetic using *trnL-F*. The phylogenetic research in *Pandanus mangalorensis* using *trnL* has also been done by Zanan & Nadaf, (2012).

Therefore, more comprehensive information in determining species identity through phylogenetic between pandan of PBG's collection using confirmation of morphological characters from vegetative organs and chloroplast genes (*trnL* and *trnL-F*) is indispensable. This information is expected to clarify the identity of the species level of PBG's *Pandanus* to support a further study on the basic conservation activities of pandan.

MATERIALS AND METHODS

Plant samples.

Samples are collections of Purwodadi Botanical Garden, Pasuruan, East Java that result of exploration from various regions in Indonesia (**Table 1**). The plant sample includes 20 pandan specimens and one specimen is *Freycinetia* as outgroup.

Table 1. Pandan Collection Specimen Purwodadi Botanical Garden

No	Specimen	Accession Number	Origin
1.	specimen (3)	P198502306	North Maluku
2.	specimen (4)	P200612116	East Java
3.	specimen (5)	P19850862	West Java
4.	specimen (6)	P19840329	East Java
5.	specimen (7)	P19820917	East Java
6.	specimen (8)	P198608255	Papua
7.	specimen (9)	P198608232	Papua
8.	specimen (10)	P200908137	South East Sulawesi
9.	specimen (11)	P2013050045	East Nusa Tenggara
10.	specimen (13)	P19821118	East Java
11.	specimen (14)	P19820232	East Java
12.	specimen (16)	P19850863	West Java
13.	specimen (17)	P198303148	East Java
14.	specimen (18)	P19850840	West Java
15.	specimen (19)	P198108145	East Java
16.	specimen (20)	P19971178	East Java
17.	<i>Freycinetia</i> sp.	P200908355	East Nusa Tenggara

DNA Isolation.

Isolation of DNA from young leaves using Genomic DNA Purification Kit (Promega), which consists of nucleus lysis solution, precipitation protein, RNase and DNA rehydration solution (10mM Tris-HCl (pH 7.4), EDM 1 mM (pH 8.0)). The method used based on the technique on the plant plus the modification of overnight incubation process at 4°C after addition of isopropanol. The qualitative DNA test used agarose electrophoresis gel method (1%) with Ethidium Bromide (EtBr) of 1 µL dissolved in 1x TBE solution. The isolated DNA samples were then visualized using a UV transilluminator with DNA ladder Sizer™

DNA Marker 1000 PLUS. Quantitative DNA used absorbance test, 260 and 280 nm (Fatchiyah et al., 2011; Hapsari et al., 2015).

Amplifikasi PCR.

The primer used are *trnL-F* forward (5'-CGAAATCGGTAGACGTACG-3') and reverse (5'-ATTTGAACTGGTGACACGA-3'), and *trnL* forward (5'-CGAAATCGGTAGACGCTACG-3') and reverse (5'-GGGGATAGAGGGACTTGAAC-3'). PCR mix 5µL, *TrnL* and *TrnL-F* Forward-Reverse primer of 1µL, and 1µL DNA samples. All materials were amplified by PCR thermal cycle tool using initial denaturation (1x) 94°C for 5 minutes; Main cycle of 35x (denaturation 94°C for 30 seconds, annealing 61°C for 30 seconds, extension 72°C for 30

seconds) and extension final (1x): 72°C for 10 minutes. The qualitative DNA test was performed using gel electrophoresis method (1.2%), Ethidium Bromide (*EtBr*) of 1 µL, then visualized. The amplified DNA samples of 30 µL were used for sequencing (Fatchiyah et al., 2011; Hapsari et al., 2015).

Data Analysis

The sequenced result data was done by homology test through BLAST on NCBI with homology level $\geq 92\%$. All sequences of sequenced (in group and out group) sequence alignment using the Clustal W program in the MEGA 6.0, with statistical program to generate the phylogeny tree (cladogram) by Maximum Parsimony (MP) method. This uses the aligned sequence by comparing sequence characters directly. The

last step is consensus the cladogram of each primer (*trn L* and *trnL-F*).

The primary data which is the result of the vegetative character description (leaves, stems and roots) and generates 50 characters which will be followed by the observation and measurement process on each character (**Table 2**). Furthermore, the measurement data results are given multistate scoring (0, 1, 2 and 3). This is intended to facilitate the grouping and analysis of data. Further assessment data were analyzed using the Maximum Parsimony (MP) method using the PAST 1.34 program to build the phylogeny tree (dendrogram). The next final stage is consensus phylogeny tree by vegetative characters (dendrogram) and molecular (cladogram).

Table 2. Morphological Characters and Multistate Scoring of Vegetative Organ.

Leaf
1. The length of apex (cm) 1 short (<3, 99); 2 mediums (4.00-5.99); 3 longs (≥ 6.00).
2. Leaf length (cm): 1 short (<99.9); 2 mediums (100-199); 3 longs (≥ 200).
3. Leaf width (cm): 1 narrow (<3.99); 2 mediums (4.00-5.99); 3 wides (≥ 6.00).
4. Leaf thickness (cm): 1 thin (<0.049); 2 mediums (0.05-0.099); 3 thick (≥ 0.10).
5. The appearance of veilet: 1 not clear; 2 sketchy; 3 clearly.
6. The number of veinlet: 1 bit; 2 mediums; 3 lots.
7. Distance between veinlet: 1 close; 2 mediums; 3 distantly.
8. Leaf upper surface: 1 glabrous; 2 spiny glabrous; 3 shiny slicks.
9. Midrib: 1 bit spiney 2 spiney along <i>the midrib</i> ; 3 others.
10. Leaf margin: 1 slightly spiny; 2 spiny along the leaf margin; 3 others
11. Size of spines on leaf margin: 1 small (<0.049); 2 mediums (0.05-0.19); 3 larges (≥ 0.2).
12. The size of spines on <i>the midrib</i> : 1 small (<0.049); 2 mediums (0.05-0.19); 3 larges (≥ 0.2).
13. Position of spines: 1 leaf margin only; 2 margin and leaf <i>midrib</i> ; 3 margin, upper surface and <i>midrib</i> .
14. Density of upper spines (cm): 0 absent; 1 close (<0.49); Medium (0.50-0.99); distantly (≥ 1.00).
15. Spines on margin density (cm): 0 absent; 1 close (<0.49); Medium (0.50-0.99); distantly (≥ 1.00).
16. Spines on <i>midrib</i> density (cm): 0 absent; 1 close (<0.49); Medium (0.50-0.99); distantly (≥ 1.00).
17. Margin spiness growth direction: 1 tip; 2 bases; 3 others.
18. Midrib spines growth direction: 1 end; 2 bases; 3 others.
19. Basal diameter of margin spines (cm): 1 small (<0.019); 2 mediums (0.020-0.029); 3 larges (≥ 0.03).
20. Basal diameter of midrib spines (cm): 1 small (<0.019); 2 mediums (0.020-0.029); 3 larges (≥ 0.03).
21. Color of margin spines: 1 light green; 2 brownish green; 3 others.
22. Color of midrib spines: 1 light green; 2 brownish green; 3 others.
23. % Closing of spines on the upper surface: 0 absent; 1 <50%; 2 ≥ 50 ; 3 100%.
24. % Closing of spines on <i>the midrib</i> : 0 absent; 1 <50%; 2 ≥ 50 ; 3 100%.
25. The depth of the leaf <i>curve</i> (cm): 1 shallow (<1.49), 2 mediums (1.50 to 2.49); 3 deeps (≥ 2.50).
26. The distance between the margin's <i>curve</i> (cm): 1 narrow (<0.99); medium (1.00-1.49); wide (≥ 3.0).
27. The distance of leaf margin to seond margin's <i>curve</i> : 1 narrow (<1.99); 2 mediums (2.00-2.99); 3 wides (≥ 3.00).
28. The existence of <i>auricle</i> : 0 absent; 1 exists.

Leaf

29. *Auricle* size: 0 absent; 1 short (<2.9); 2 mediums (3.0-4.9); 3 longs (≥ 5.0).
 30. *Auricle* Width (cm): 0 absent; 1 short (<2.9); 2 mediums (3.0-4.9); 3 wides (≥ 5.0).
 31. The existence of spines on the margin of *auricle*: 0 Absent: 1 exist.
 32. Frequency of spines on *auricle*: 0 absent; 1 close (<0.049); 2 mediums (0.05-0.099); 3 rare (≥ 0.1).
 33. Length of *auricle* spines: 0 absent; 1 short (<0.049); 2 mediums (0.05-0.099); 3 longs (≥ 0.1)
 34. The Color of *auricle*: 0 absent, 1 white; 2 greens; 3 others.
 35. Length of sheath (cm): 1 short (<1.9); 2 mediums (2.0-3.9); 3 longs (≥ 4.0).
 36. Width of sheath (cm): 1 narrow (<3.9); 2 mediums (4.0-5.9); 3 wides (≥ 6.0).
 37. Sheath position to stem: 1 does not cover; 2 covering the sheath; 3 *overlapping*.
 38. Apex shape 1 acute; 2 acuminate; 3 others.
 39. Leaf shape: 1 linnear; 2 oblongata; 3 others
 40. Leaf upper surface color: 1 light green; 2 dark green; 3 others.
 41. Leaf bottom surface color: 1 light green; 2 dark green; 3 others
 42. Leaf scent: 0 absent; 1 less fragrance; 3 fragrances.
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Stem

43. Stem length (cm): 1 short (<0.99); 2 mediums (1.00-1.99); 3 longs (≥ 2.0).
 44. Stem diameter (cm): 1 small (<1.99); 2 mediums (1.00-1.99); 3 larges (≥ 2.0).
 45. Stem surface condition: 1 former leaf sitting; 2 thorns; 3 others.
 46. Stem color: 1 light brown; 2 browns; 3 others.
 47. Stem growth Direction: 1 upright; 2 spreads; 3 others.
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Root

48. Character of roots above the ground: 1 prop roots; 2 lianas; 3 others.
 49. Root surface above ground: 1 thorny/nodule; 2 smooths; 3 others.
 50. Tree height (cm): 1 short (<99); 2 mediums (100-199); 3 high (≥ 200).
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RESULT AND DISCUSSION

DNA Amplification

Based on the results of isolation and DNA amplification of two primers, shows that only some specimens are successfully well amplified. Primer *trnL*, there were 20 OTUs that were successfully amplified except spesimen (2) (**Figure 1**).

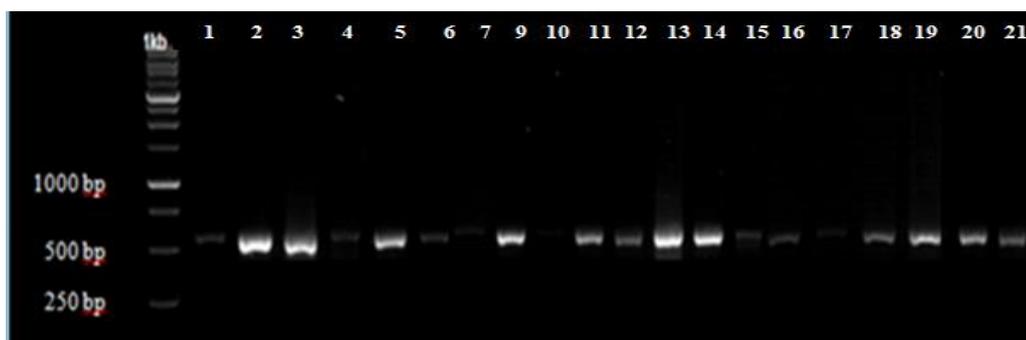


Figure 1. PCR product of primer *trnL*: 1. spesimen (12); 2. spesimen (19); 3. *Freycinetia*; 4. spesimen (18); 5. spesimen (13); 6. spesimen (17); 7. spesimen (15); 9. spesimen (4); 10. spesimen (1); 11. spesimen (14); 12. spesimen (3); 13. spesimen (5); 14. spesimen (6); 15. spesimen (16); 16. spesimen (17); 17. spesimen (8); 18. spesimen (10); 19. spesimen (7); 20. spesimen (20); 21. spesimen (11).

Primer *trnL-F*, there are 18 OTUs that can be amplified except spesimen (1), spesimen (7), and spesimen (12) (**Figure 2**). OTU that have thick band can be analyze to sequencing stage. In this study used each of 17 of 21 OTU to be analyzed kinship relationship.

Variasi Sekuen DNA

Polymorphisms analysis of 450 nucleotide based on primer *trnL* of 17 OTU that has been alignmented. The alignment results indicate that as many as 180 (40%) characters are conserved areas (**Table 3**). The conserved area is an area that has a nucleotide bases position that does not changes, so it is a primitive character (plesiomorph) (Hidayat & Pancoro, 2008).

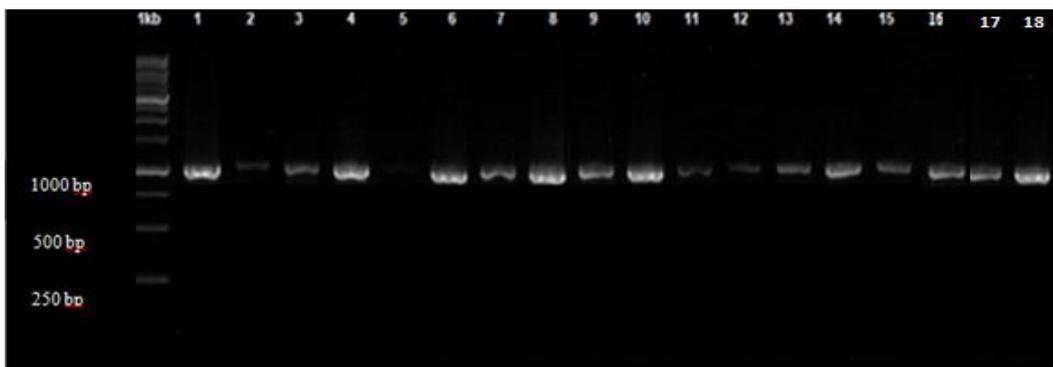


Figure 2. PCR product of primer *trnL-F*: 1. spesimen (19); 2. *Freycinetia* sp.; 3. spesimen (18); 4. spesimen (13); 5. spesimen (17); 6 spesimen (2); 7. spesimen (4); 8. spesimen (14); 9. spesimen (3); 10. spesimen (5); 11. spesimen (16); 12. spesimen (6); 13. spesimen (8); 14. spesimen (9); 15. spesimen (10); 16. spesimen (7); 17. spesimen (20); 18. spesimen (11).

Polymorphic variables as 198 (44%) characters and 72 (16%) are *gaps* or missing nucleotide bases. *Singleton* which is auupperomorph variation of nucleotide bases sequences of 190 characters and parsimony informative are 8 characters. *Singleton* variation is a nucleotide bases that change that at least once in a single OTU (Hidayat & Pancoro, 2008). The Results of polymorphism analysis using *trnL-F* obtained 658 nucleotide bases of 17 OTU. The position of conserved bases is 586 (89.05%) with character of polymorphic base position is 22 (3.34%) characters and 50 (7.59%) characters are missing base position (*gaps*) in the sequence. As for the variation *singleton*, 14 characters and 8 characters are parsimony informative.

Table 3. The Results of Polymorphism Analysis of DNA Sequences By 2 Primers

Primers	Total Character by Alignment	Total Character Conservation	Total Character <i>Singleton</i>	Total Character Informative Parsimony
<i>trnL</i>	450	180	190	8
<i>trnL-F</i>	658	586	14	8

Comparison of Phylogenetic Tree Consensus Based on Morphological and Molecular Character.

A phylogenetic tree consensus comparison (**Figure 3**) results in a grouping of kinship relationships based on morphological and molecular characters. The Grouping is divided into two major clades. Both phylogenetic trees appear to be similar and mutually supportive. The difference is clearly visible on the composition of the specimen-forming groups (clade). Based on its forming characters, clade I in dendrogram has a similar composition to spesimen cladogram clade II in which are the spesimen (2), (9), (12), (13), (17), and (18). Otherwise, clade II on the dendrogram has the character and composition

of the same specimen that forming clade I. Differences in the composition of the specimen of pandan does not significantly affect the specimen grouping based on morphological and molecular. In fact, the two phylogenetic trees support each other.

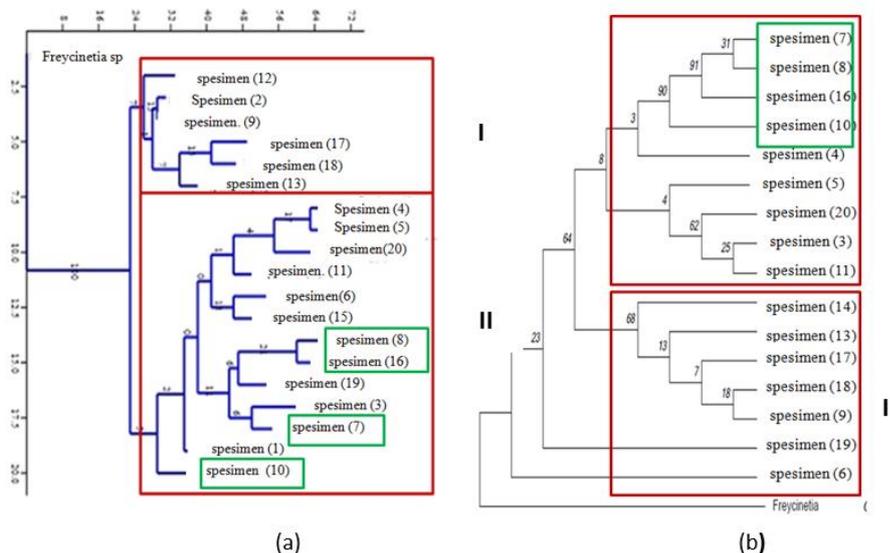


Figure 3. Phylogenetic tree: (a) morphology dan (b) molekuler (*trnL* dan *trnL-F*).
Note: (red) *Pandanus* grouping; Green: existence of *Benstonea*.

Phylogenetic by Molecular.

Based on the cladogram using MP analysis method (Figure 3b), seen the establishment of two major clades. Clade I consists of 9 OTU, clade II consists of 5 OTU. Both clades are supported by different bootstrap values on each branch. In addition, there are 3 monophyletic branches, the first monophyletic with bootstraps value is 31, which is between the spesimen (7) and spesimen (8). The autopomorphs formed between the two are 1 transversion, 4 transitions, and 2 insertions total is 17 mutations. According to Hapsari et al. (2015) the number of autopomorphs indicates the adaptation of the individu to the environment. This monophyletic has highest *bootstrap* value and supported by the genetic distance of 0.007 (Appendix 1). Second monophyletic has a *bootstrap* value of 25, are between the spesimen (3) and spesimen (11). Both OTU were formed by autopomorphs of 2 transversions, 4 transitions, and 5 insertions with a total

mutation of 52 with a genetic distance of 0.002. The third monophyletic is between the spesimen (16) and (9) with autopomorphs of 2 transversions, 4 transitions, and 3 insertions with a total of 55 mutations. The genetic distance formed by the third monophyletic is 0.012. Wijayanto et al. (2013) said that the greater value of genetic distance indicates a far relationship.

Phylogenetic by Morphology Characters

Based on the results of phylogenetic trees using vegetative organs characters of 21 OTU seen formation of two major clades (Figure 3a). Both clades are formed based on morphological characters of vegetative organs. There are 3 characters that can be differentiating in pandan grouping in morphology.

Spines Position on Leaf.

Results of a family tree using the MP, showed two major clades, namely, the existence of a thorn in the side edge of the leaf and bottom of main leaf bone (midrib).

Appendix 1. Consensus Genetic Distance of 17 OUT Pandan Purwodadi Botanical Garden Collection.

No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	Freycinetia_sp.		0.003	0.003	0.003	0.003	0.004	0.005	0.003	0.004	0.003	0.003	0.003	0.086	0.009	0.003	0.004	0.003
2	spesimen_(3)	0.009		0.001	0.002	0.002	0.003	0.004	0.003	0.003	0.001	0.002	0.002	0.081	0.008	0.002	0.003	0.002
3	spesimen_(4)	0.008	0.002		0.001	0.002	0.003	0.003	0.002	0.002	0.001	0.002	0.001	0.081	0.008	0.001	0.002	0.001
4	spesimen_(5)	0.009	0.003	0.001		0.002	0.003	0.004	0.003	0.002	0.002	0.002	0.002	0.081	0.008	0.002	0.002	0.001
5	spesimen_(6)	0.011	0.005	0.005	0.006		0.003	0.004	0.003	0.003	0.003	0.002	0.002	0.085	0.008	0.002	0.002	0.002
6	spesimen_(7)	0.016	0.010	0.010	0.011	0.009		0.002	0.004	0.002	0.004	0.003	0.003	0.080	0.009	0.003	0.004	0.003
7	spesimen_(8)	0.019	0.013	0.011	0.012	0.016	0.007		0.004	0.003	0.004	0.004	0.004	0.077	0.010	0.004	0.005	0.004
8	spesimen_(9)	0.012	0.008	0.007	0.008	0.011	0.016	0.018		0.003	0.003	0.002	0.002	0.085	0.008	0.002	0.003	0.003
9	spesimen_(10)	0.013	0.007	0.005	0.006	0.008	0.005	0.008	0.012		0.003	0.003	0.003	0.080	0.009	0.003	0.003	0.002
10	spesimen_(11)	0.010	0.002	0.002	0.003	0.007	0.012	0.013	0.009	0.007		0.002	0.002	0.081	0.008	0.002	0.003	0.002
11	spesimen_(13)	0.008	0.005	0.004	0.005	0.007	0.012	0.015	0.006	0.009	0.006		0.001	0.082	0.008	0.001	0.003	0.002
12	spesimen_(14)	0.010	0.004	0.002	0.003	0.007	0.012	0.013	0.005	0.007	0.004	0.002		0.082	0.008	0.000	0.003	0.002
13	spesimen_(16)	0.320	0.308	0.309	0.311	0.315	0.305	0.300	0.319	0.308	0.312	0.314	0.312		0.096	0.082	0.085	0.081
14	spesimen_(17)	0.046	0.039	0.039	0.040	0.042	0.046	0.049	0.040	0.043	0.039	0.038	0.037	0.333		0.008	0.009	0.008
15	spesimen_(18)	0.010	0.004	0.002	0.003	0.007	0.012	0.013	0.005	0.007	0.004	0.002	0.000	0.312	0.037		0.003	0.002
16	spesimen_(19)	0.012	0.007	0.005	0.006	0.004	0.013	0.016	0.012	0.010	0.007	0.008	0.007	0.315	0.042	0.007		0.002
17	spesimen_(20)	0.008	0.004	0.002	0.003	0.005	0.010	0.013	0.009	0.006	0.004	0.004	0.004	0.312	0.040	0.004	0.006	

Those characters are owned by several specimens such as spesimen (1), spesimen (2), spesimen (3), spesimen (4), spesimen (5), spesimen (9), spesimen (12), spesimen (13), spesimen (17), spesimen (18) and spesimen (20). spesimen (6), spesimen (7), spesimen (8), spesimen (10), spesimen (11), spesimen (15), spesimen (16), and spesimen (19) has the character trait spine on the margin, *the midrib* and the upper surface of the leaves. Spine on pandan is a character that is easily recognizable, as well as a distinguishing character to the spesies level (Stone, 1983; Rahayu & Handayani, 2008). (Figure 4).

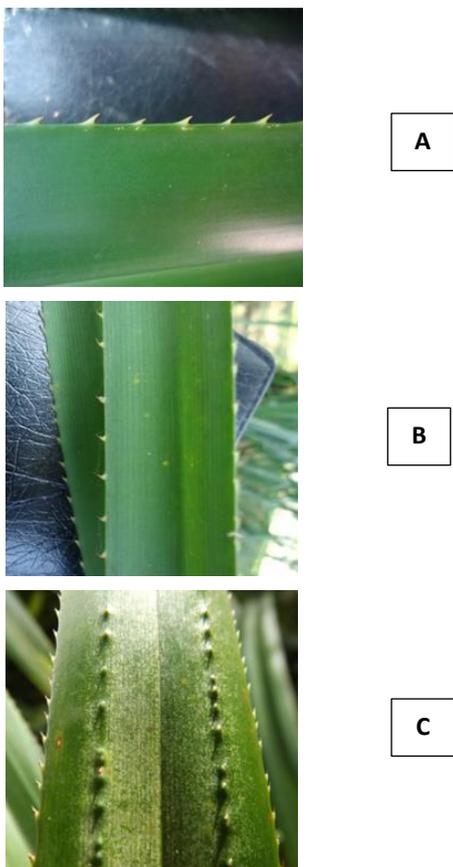


Figure 4. Spine position (a) leaf margin; (b) leaf midrib; (c) leaf upper surface

Apex Shape.

Pandan has two forms of the tip of the leaf that can be a distinguishing character in grouping of several types of pandanus, that

is the pointed tip and tapering. The spesimen (2), spesimen (6), spesimen (9), spesimen (13), spesimen (14), spesimen (15), spesimen (16), spesimen (17), and spesimen (18) have the apex. The acuminate apex is owned by spesimen (3), spesimen (4), spesimen (5), spesimen (11), and spesimen (20). According to Judd WS et al. (2002), the acute shape of the apex is more modern than the acuminate form. It is possible that the *Pandanus* has the apex shape is a more modern spesimen. (Figure 5)

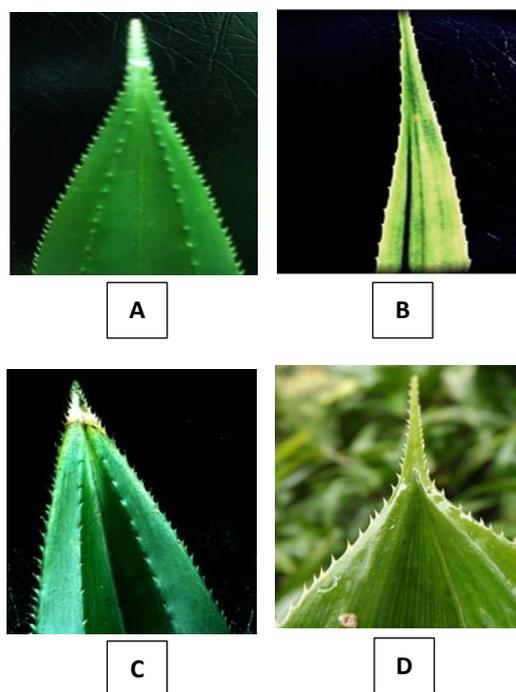


Figure 5. Apex shape (a) acuminate; (b) acute; (c) Slightly folded on the apex leaf; (d) mucronate.

Root Surface Condition above Soil.

There are 2 characters of Pandan root surface above soil, which are smooth and thorned/ noded/ if touched with hand. Spesimen (2), spesimen (9), spesimen (12), spesimen (13), spesimen (17), and spesimen (18) have smooth root surfaces. While the roots with thorn or nodules characters are owned by several *Pandanus* such as in spesimen (6), spesimen (9), spesimen (13), spesimen (14), spesimen (17), spesimen (18) and spesimen (19).

Based on the phylogenetic tree using the MP, clade I have the character of a spine on the leaf margin and the leaf *midrib*; the shape of apex is acute and the smooth root surface. Clade II was formed on the character of the opposite, spines on the leaf margin, and the upper surface of the leaf *midrib* except the spesimen (4), (5) and (20) which has spines on the edges and the leaf *midrib* only. The shape of the tip tapered leaves except the spesimen (12) having a mucronate *shape*. The last character forming clade II have nodules root surface, while the thorn root characters appear on the spesimen (17) (Figure 6).

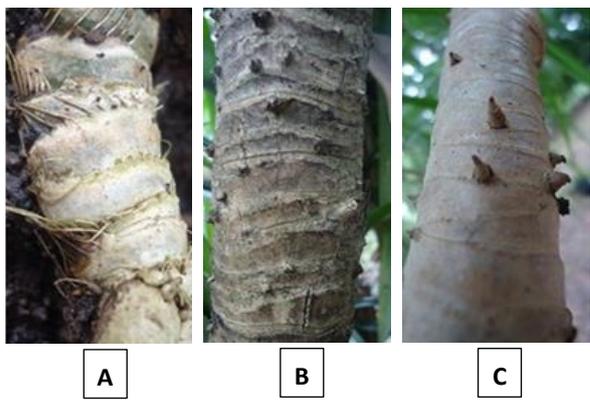


Figure 6. Roots condition (a) former leaf sitting; (b) nodules; (c) thorny.

Position Estimation of Taxon Pandan of KRP Collection.

Based on the homology test results on 17 specimens of pandan collection of PBG using chloroplast genes (*trnL* and *trnL-F*),

there are two differences between *Pandanus* and *Benstonea*. Both genera have the value of the similarity identification $\geq 98\%$ (Table 4). These results support the monophyletic branch of a phylogenetic tree formed on the basis of molecular character. Based on these results, both monophyletic specimens (7 and 8) suggest to be the same specimen that is *Benstonea lauterbachii*, with the character of vegetative organ similar to the *Pandanus* but with the acute apex shape and slightly tightened on both sides. The second monophyletic between specimen (3) and (11) shows the name of the same species that is *Pandanus aimirikensis*. Those species are supported by morphological characters such as the position of spine on leaf margin, upper surface, and the leaf *midrib*; acuminate apex shape and smooth root surface. While the third monophyletic between the spesimen (9) and (18) has two possible names, they are *Pandanus cookies* and *Pandanus baptisii*. That species has the opposite characters i.e., the position of spine on the leaf margin and the leaf *midrib*; acuntus apex shape; a nodule root surface. However, the four names of the species are not the names of the Indonesian endemic *Pandanus* species. This is because few research about Indonesian *Pandanus* using molecular techniques and the sequences in the gene bank database that is homologous with *Pandanus* in Indonesia.

Table 4. Homology Test of Pandanus Specimen Collection of Purwodadi Botanical Garden.

No.	Specimen	BLAST Result		Gene Bank Code	
		trn L	trn L-F	trn L	trn L-F
1	Spesimen (3)	<i>Pandanus aimirikensis</i>	<i>Pandanus aimirikensis</i>	KJ681532.1	KJ681503.1
2	Spesimen (4)	<i>Pandanus kanahirae</i>	<i>Pandanus tectorius</i>	KJ681605.1	FJ194471.1
3	Spesimen (5)	<i>Pandanus furcatus</i>	<i>Pandanus furcatus</i>	KJ681502.1	KJ681502.1
4	Spesimen (6)	<i>Pandanus brachyspathus</i>	<i>Pandanus brachyspathus</i>	KJ681517.1	KJ681517.1
5	Spesimen (7)	<i>Benstonea lauterbachii</i>	<i>Benstonea lauterbachii</i>	KJ681568.1	KJ681568.1
6	Spesimen (8)	<i>Benstonea lauterbachii</i>	<i>Benstonea lauterbachii</i>	KJ681568.1	KJ681568.1
7	Spesimen (9)	<i>Pandanus cookii</i>	<i>Pandanus veitchii</i>	KJ681633.1	AF293104.1
8	Spesimen (10)	<i>Benstonea monticola</i>	<i>Benstonea monticola</i>	KJ681562.1	J681562.1

No.	Specimen	BLAST Result		Gene Bank Code	
		trn L	trn L-F	trn L	trn L-F
9	Spesimen (11)	Pandanus aimirikensis	Pandanus aimirikensis	KJ681532.1	KJ681503.1
10	Spesimen (13)	Pandanus cookii	Pandanus sp.	KJ681633.1	KJ681596.1
11	Spesimen (14)	Pandanus cookii	Pandanus sp.	KJ681633.1	KJ681596.1
12	Spesimen (16)	Benstonea lauterbachii	Benstonea lauterbachii	KJ681568.1	KJ681568.1
13	Spesimen (17)	Pandanus cookii	Pandanus tectorius	KJ681633.1	KJ681500.1
14	Spesimen (18)	Pandanus cookii	Pandanus baptisii	KJ681633.1	KJ681499.1
15	Spesimen (19)	Pandanus brachyspathus	Pandanus brachyspathus	KJ681517.1	KJ681517.1
16	Spesimen (20)	Pandanus amaryllifolius	Pandanus amaryllifolius	KJ681507.1	KJ681507.1
17	Freycinetia sp.	Freycinetia multiflora	Freycinetia banksii	KJ681552.1	KJ681531.1

Note. Thick marked (gray highlights) is a *Benstonea*.

The occurrences *Benstonea* in the homology test results are outside the research hypothesis. *Benstonea* is originally a member of the genus *Pandanus* subgenus *Acrostrigma* (Kurz) BC Stone by morphological character, but there is still confusion. Character of vegetative organs of *Benstonea* are not much different from the characters of *Pandanus*. Differences showed in the generative organ that can be strong evidence to separate both genus. The characters are the *carpels* always free; stigma can be found in the abaxial and sharp; there is an ovule in each drupe; the stamen reduces to one or connate and free at the base of the stamen (Callmander et al., 2013). *Pandanus* has connate or free carpel; stigma can be found in adaxial of various forms but not sharp; there is drupe of several ovules; and there are connate in one stamen (Stone, 1983). The appearance of another genus is very possible to happen considering both them are widespread throughout Indonesia. *Benstonea* mostly found in Borneo and some in Java and Papua island (Indonesia) (Callmander et al., 2012) and *Pandanus* who growth in almost forests, beaches and mountains in Indonesia (Rahayu & Handayani, 2008).

CONCLUSION

Pandanus family is a group of a plant that is complex and complicated regarding

relationship. The identification of plant relationship using monophyletic specimen which is forming from the consensus of phylogenetic tree, based on the morphology and molecular characteristics, results in four suggest names which came from three monophyletic branches which are *Benstonea lauterbachii*, *Pandanus aimirikensis*, *Pandanus cookie* and *Pandanus baptisii*. Those species name are suspected to come from the same species but different accession region. Surprisingly, the specimen which was initially identified as *Pandanus*, was known to be the new genus of *Benstonea* based on the homology test of its DNA sequence by this research. It has not been revealed before by PBG researcher. This result supported the previous research of Callmander et al. (2012) about the existence of *Benstonea* in Indonesia. The use of primer of *trnL* and *trnL-F* seemed to not informative enough to reveal the position of pandan infraspecies taxon. Thus, the further phylogenetic analysis needs to using the other piece of DNA as the molecular marker to clarify the position of *Pandanus* taxon at PBG collection.

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