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Genetic Diversity and Relationships of *Phalaenopsis* Based on the *rbc*L and *trn*L-F Markers: *In Silico* Approach

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Abstract. *In silico* is the more comprehensive and applicable approach in supporting, both conservation and breeding programs of germplasm. The study aimed to analyze and determine the genetic diversity and relationships of 24 species of *Phalaenopsis* using two DNA barcoding markers, namely the *rbc*L and *trn*L-F, by *in silico* approach. All sequences of these *markers* were collected randomly from the NCBI website and analyzed using several softwares and methods, such as ClustalW and MultAlin for multiple sequence alignments and MEGA-X to determine its genetic diversity and relationships. Specifically, the genetic diversity was determined using a nucleotide diversity index and their relationships by the Maximum Likelihood method. The results showed that *Phalaenopsis has* a low genetic diversity of 0.24, 0.32, and 0.19, respectively. The phylogenetic analysis revealed that this orchid separated into five (for the *rbc*L), six (*trn*L-F), and seven clades (a combined one), where the closest relationship is shown by *P. amboinensis* vs. *P. venosa*, whereas the farthest by *P. gibbosa* vs. *P. doweryensis*, *P. stuartiana* vs. *P. micholitzii*, and *P. celebensis* vs. *P. pulchra*. The results have novel information on the diversity and relationships of *Phalaenopsis* on *the in silico* approach. Thus, our findings might be used in supporting the conservation and breeding program of *Phalaenopsis*, both locally and globally.

Key words: DNA barcoding; genetic diversity; *in silico*, *Phalaenopsis*; phylogenetic analysis

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INTRODUCTION

Phalaenopsis, commonly known as moth orchid (Tsai et al., 2012) is the most popular orchid genus in the world (Chen et al., 2013b; Deng et al., 2015; Hsu et al., 2018). The popularity of this orchid is mainly related to the characteristics of the flowers it has, both shape, color, scent, and a long-lasting blossom (Hsu et al., 2011). Besides, *Phalaenopsis* is fast-growing and flowering, has a relatively short juvenile period, and easy to control at the flowering stage (Chen & Lin, 2012). Firgiyanto et al. (2016) reported that *Phalaenopsis* also has resistance and the ability to flower under unfavorable conditions.

Globally, *Phalaenopsis* consists of about 66 endemic species that are scattered mainly in the western and southeastern Asian regions (Hinsley et al., 2018; Liu et al., 2016), covering Sri Lanka, India, Himalayas, China, Tibet, Philippines, Andaman Islands, Taiwan, Indonesia, and Papua New Guinea (Chen et al., 2013b; Deng et al., 2015; Rahayu et al., 2015), including northern Australia (Tsai et al., 2010; Tsai, 2011). According to Deng et al. (2015) and Tsai et al. (2010), the highest *Phalaenopsis* diversity was found in Indonesia and Philippines. Especially in Indonesia, there are more than 20 species of *Phalaenopsis* scattered in several large islands, including Sumatra, Java, Kalimantan, Nusa Tenggara, Sulawesi, Maluku, and Papua (Fatimah & Sukma, 2011; Rahayu et al., 2015).

Unfortunately, most of the *Phalaenopsis* species are currently very difficult to find in the wild, even among them are in the threatened category (Zhang et al., 2018). Deforestation, habitat destruction, overexploitation, and illegal trading, as well as other environmental impacts, are the major causes of the decline in the *Phalaenopsis* population in the wild (Fatimah & Sukma, 2011; Luo et al., 2014; Zahara & Win, 2019). Hence, the preservation, breeding, and analysis of genetic diversity of *Phalaenopsis* orchids are very urgent to employ.

For decades, analysis of genetic diversity, including orchids, has been carried out conventionally, using morphological markers (Kwon et al., 2017). However, these markers are greatly influenced by environmental factors and plant growth phases, so they are time-consuming (Kwon et al., 2017; Nadeem et al., 2018). Several molecular markers have used to study the genetic diversity of *Phalaenopsis*, namely RAPD (Goh et al., 2005; Niknejad et al., 2009), AFLP (Chang et al., 2009), and SSR (Chung et al., 2017; Fatimah & Sukma,

2011; Tsai et al., 2015b). However, these markers also have weaknesses, such as very subjective, and the results of the analysis are less accurate (Lee et al., 2017).

Currently, chloroplast DNA (cpDNA), known as DNA barcoding markers can be used to determine the genetic diversity and relationship of germplasm, including orchids (Jheng et al., 2012; Tsai et al., 2012). These markers have advantages over some of the previously mentioned, such as faster and more accurate in determining the genetic diversity of germplasm (Lee et al., 2017; Li et al., 2015; Singh et al., 2017). The Consortium for the Barcode of Life's or CBOL (2009) have recommended several DNA barcoding markers, two of these are the *rbc*L and *trn*L-F.

The *rbc*L is a coding region of cpDNA that has a low rate of polymorphism or mutation. However, this marker have generated a high quality output of sequence and a high universality of primer, then easy to aligned across various plant taxa (Dong et al., 2014). Furthermore, the *trn*L-F is a non-coding region of cpDNA with a number of structural mutations found, especially the insertions-deletions (indels). Hence, it can be used as a reliable genetic marker in population genetics and plant systematics (Chen et al., 2013a). This marker has also a conserve region that provides the opportunity to create universal primers for various plant taxa (Taberlet et al., 1991). The combination of these two (*rbc*L and *trn*L-F) markers have successfully applied for identification of NW-European fern (de Groot et al., 2011).

This study aimed to analyze the genetic diversity and relationship of 24 species of *Phalaenopsis*, based on the *rbc*L and *trn*L-F markers, by *in silico* approach. It means we have collected and used those markers from the GenBank or the National Center for Biotechnology Information (NCBI). According to Mascher et al. (2019), this institution provides a comprehensive database of nucleotide sequences or gene descriptions that are freely accessed. Hence, such a study does not require high costs and is applicable to support germplasm conservation, breeding, and cultivation programs (Mursyidin & Makruf, 2020). In other words, our findings may be usable as a reference in supporting the conservation and breeding programs of *Phalaenopsis*, both locally and globally.

METHODS

Data collection

The *rbc*L and *trn*L-F sequences of 24 *Phalaenopsis* species were collected randomly from the GenBank or NCBI website (https://www.ncbi.nlm.nih.gov). All sequences of both regions (Table 1) were then saved into FASTA or Notepad (text) format.

Table 1. The *rbc*L dan *trn*L-F sequences of 24 species of *Phalaenopsis* used in the study

Species	GenBank Accession Number		Nucleotide Length (bp)		
	*rbc*L	*trn*L-F	*rbc*L	*trn*L-F	Combined
P. amabilis	AY389440.1	AY273653.1	706	1126	1834
P. amboinensis	AY389422.1	AY265743.1	698	585	1283
P. Aphrodite	AY389441.1	AY273652.1	706	1117	1825
P. borneensis	AY389386.1	AY265747.1	687	584	1271
P. braceana	AY389405.1	KJ733669.1	688	1047	1737
P. celebensis	AY389432.1	AY265799.1	698	590	1288
P. chibae	AY389412.1	AY273667.1	718	1078	1798
P. cornu-cervi	AY389408.1	AY273664.1	687	1113	1802
P. doweryensis	AY389395.1	AY273627.1	687	1094	1781
P. equestris	AY389430.1	AY273651.1	704	1094	1798
P. fuscata	AY389388.1	AY273647.1	669	1098	1767
P. gibbose	AY389427.1	AY273680.1	692	1113	1807
P. gigantea	AY389394.1	AY273625.1	677	1114	1791
P. inscriptiosinensis	AY389423.1	AY273673.1	699	1111	1812
P. lowii	AY389439.1	KJ733671.1	681	1059	1742
P. micholitzii	AY389438.1	AY265771.1	696	588	1284
P. parishii	AY389402.1	AY265774.1	682	571	1255
P. philippinensis	AY389411.1	AY273656.1	705	1125	1832
P. pulchra	AY389399.1	AY273639.1	699	1096	1795
P. stuartiana	AY389403.1	AY273654.1	705	1121	1828
P. sumatrana	FJ460418.1	AY273677.1	700	1121	1823
P. venosa	AY389406.1	AY273642.1	698	1107	1807
P. violacea	AY389397.1	AY265796.1	707	569	1278
P. wilsonii	AY389385.1	AY265787.1	688	568	1258

Multiple sequence alignment

All sequence datasets of the *rbc*L and *trn*L-F of *Phalaenopsis* were aligned using ClustalW (Kumar et al., 2018) and MultAlin (Mitchell, 1993). The multiple alignments analyses were also conducted for a combined sequence. At this stage, the conserve region and/or polymorphic sites can be observed in both sequences.

Analysis of genetic diversity and their relationships

The level of genetic diversity of 24 species of *Phalaenopsis* was determined by the nucleotide diversity index (π) with the categories: 0.1 to 0.4 is low, 0.5 to 0.7 is medium, and 0.8-2.00 is high (Nei & Li, 1979). The phylogenetic relationship of germplasm was analyzed using the Maximum Likelihood method and evaluated by a bootstrap analysis for 1,000 replicates (Lemey et al., 2009). All analyses were conducted using the assistance of MEGA-X software (Kumar et al., 2018). Other parameters, such as the number of polymorphic sites (*S*), transition/transversion bias value (*R*), and Tajima's neutrality test (*D*) were also determined using this software (Kumar et al., 2018).

RESULTS AND DISCUSSION

Genetic diversity and mutational events

Phalaenopsis has unique characteristics of the *rbc*L (Figure 1) and *trn*L-F (Figure 2) sequences. In general, both markers are equipped by a conserve region and some mutational evens, both substitutions and insertions-deletions (indels). Following Figure 1 and 2, a conserve region of both genes showing in bases with red color, whereas some mutational events, such as substitutions and insertions-deletions or indels, showing in green and orange rectangle, respectively. At a glance, following these two figures, the mutational events of *trn*L-F are relatively higher than the *rbc*L. Further information about the sequence characteristics of these two regions, including their mutational events and their specific loci are shown in Table 2.

Based on the Table 2, the *Phalaenopsis* has different of nucleotide length, both for the *rbc*L and *trn*L-F. In this case, the *rbc*L has a range of nucleotides of 669-718 bp, whereas the *trn*L-F has 568-1126 bp. According to CBOL (2009), the *rbc*L has a complete sequence, including approximately 1400 nucleotides coding for the large subunit protein, but the length varies slightly among flowering plants (Angiosperm). Singh and Banerjee (2018) reported that this region has an intergenic spacer with 600-800 nucleotides. Similarly, an entire sequence region of the *trn*L-F has also reported approximately of 1400 bp (Quandt et al., 2004).

Furthermore, there are a different number of polymorphic sites (*S*) and transition/transversion bias values (*R*) on the *rbc*L and *trn*L-F regions of *Phalaenopsis*. In general, the *rbc*L has a higher number of polymorphic sites (62 loci) than the *trn*L-F (59 loci). However, the *rbc*L has a relatively lower in transition/transversion bias values (0.40) than the *trn*L-F (0.42) (Table 2). According to Stoltzfus and Norris (2015), this bias can be described as a ratio of differences, which makes the probable effect a complex function of the degree of sequence divergence.

Table 2. Genetic information of the *rbc*L, *trn*L-F, and combined sequences of *Phalaenopsis,* including their nucleotide diversity*

Parameter	Sequence		
	*rbc*L	*trn*L-F	Combined
Range of sequence length (bp)	669-718	568-1126	1255-1834
Number of polymorphic sites (*S*)	62	59	117
Transition/transversion bias value (*R*)	0.40	0.42	0.47
Nucleotide diversity (π)	0.24	0.32	0.19
Tajima's neutrality test (*D*)	-1.173337	-2.765724	-1.446696

*Based on Kimura two-parameter model (Kumar et al., 2018)

Figure 1. The characteristic of *rbc*L sequences of *Phalaenopsis* showing a conserved region (red color) and some mutational events, such as substitutions (green rectangle) and insertions-deletions or indels (orange rectangle)

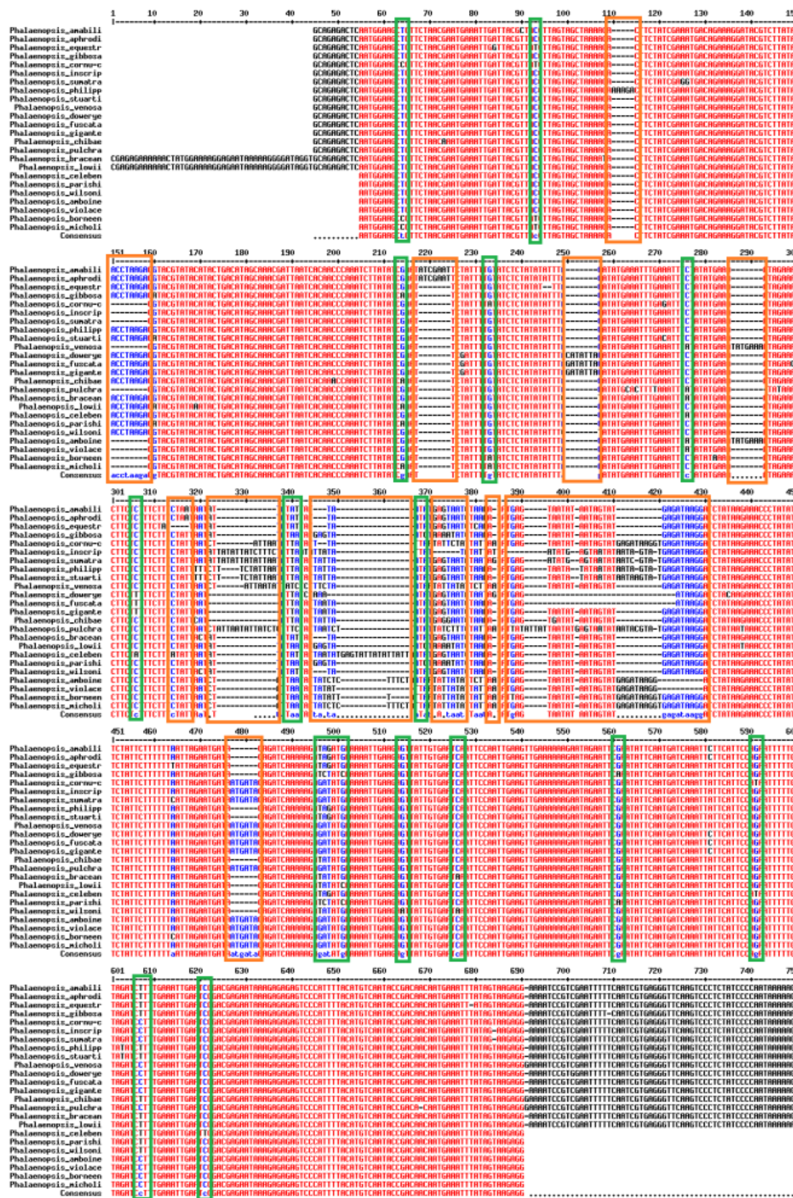

Figure 2. The characteristic sequence of *trn*L-F of *Phalaenopsis* showing a conserved region (red color) and some mutational events, such as substitutions (green rectangle) and insertions-deletions or indels (orange rectangle)

In this study, all mutations event, mainly substitutions (transition and transversion), also indels (insertion and deletion) are found in the region of the *rbc*L and *trn*L-F of *Phalaenopsis*. According to Aloqalaa et al. (2019), transitions are more often found in sequences than transversions. In other words, a pattern where nucleotide transitions are found several folds over transversions is common in molecular evolution (Stoltzfus & Norris, 2015).

Conceptually, mutations, both substitutions and indels, are therefore tend to cause changes in the biochemical properties of amino acids or the protein products (Keller et al., 2007). According to Flint-Garcia (2013), mutations are permanent changes that are inherited in the genes or nucleotide sequences

(genome) of an organism, and it can affect a single nucleotide (point mutation) or some that are close to each other (segmental mutation). The Tajima's neutrality test revealed that *Phalaenopsis* has an overage of low-frequency polymorphisms relative to expectancy, indicating population size expansion (e.g., after a bottleneck or a selective sweep) and/or purifying selection, because all sequences have negatives of D value (D<0) (Tajima, 1989).

Following Govindaraj et al. (2015), mutations are an initial step in establishing the primary population for natural selection and an integral part of evolution and genetic diversity. In other words, this phenomenon is the main factor giving rise to genetic diversity (Frankham et al., 2004). Hence, mutation and genetic diversity are two interrelated things. In this case, based on the Nei's (1979) category, *Phalaenopsis* shows a low level of genetic diversity, both for the *rbc*L (0.24) and *trn*L-F (0.32), as well as a combined sequence (0.19) (Table 2). According to Acquaah (2012), information on this diversity is valuable for future breeding and conservation programs, particularly in developing new superior cultivars.

Phylogenetic relationships

The maximum likelihood analysis shows that *Phalaenopsis* has a complicated relationship. This complexity can be seen from the clades generated by each sequence used. Based on the *rbc*L region, this orchid was separated into five main clades (Figure 3), where the very closely relationship shown by three pairs of *Phalaenopsis*, namely *P. philippinensis* vs. *P. stuartiana*; *P. amboinensis* vs. *P. venosa*; *P. sumatrana* vs. *P. inscriptiosinensis* with a similarity coefficient of 99.71. Whereas a very far related shown by *P. gibbosa* vs. *P. doweryensis* at a similarity of 91.73 (Table Supplementary 1).

Following the *trn*L-F, this orchid was separated into six main clades (Figure 4), where a very close related shown by *P. venosa* vs. *P. amboinensis*; *P. parishii* vs. *P. gibbosa* (similarity of 99.99) and a very distantly (85.82) by *P. stuartiana* vs. *P. micholitzii* (Table Supplementary 2). Furthermore, a combined sequence of both regions has separated *Phalaenopsis* into seven main clades (Figure 5), where *P. venosa* and *P. amboinensis* are a closest relationship with a coefficient similarity of 99.84, whereas the fartest shown by *P. celebensis* and *P. pulchra* (90.12) (Table Supplementary 3).

Based on the *rbc*L and *trn*L-F markers, as well as a combined one, most of the *Phalaenopsis* species are grouped into a relatively similar clade. For example, *P. celebensis*, *P. amabilis*, *P. aphrodite*, *P. equestris*, *P. philippinensis*, and *P. stuartiana* are included into a similar large member based on these three sequences (Table 3). However, there is an exception, specifically for *P. lowii* which grouping into the similar clades for *rbc*L and a combined sequence with *P. braceana* and *P. wilsonii*, and separate from these two species, but joined together with *P. chibae*, *P. gibbosa* and *P. parishii* (Table 3).

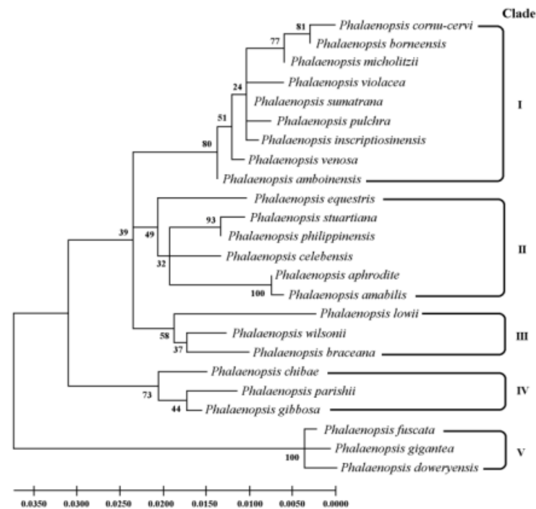

Figure 3. Phylogenetic relationship of *Phalaenopsis* based on the *rbc*L sequence. Values on the internal nodes of phylogram indicate a bootstrap analysis with 1,000 replicates

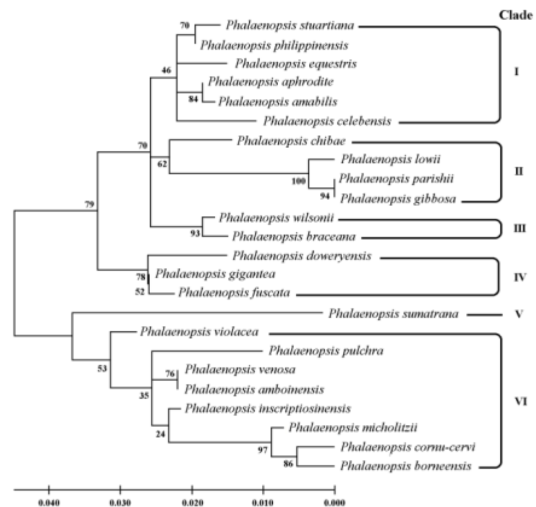

Figure 4. Phylogenetic relationship of *Phalaenopsis* based on the *trn*L-F sequence. Values on the internal nodes of phylogram indicate a bootstrap analysis with 1,000 replicates

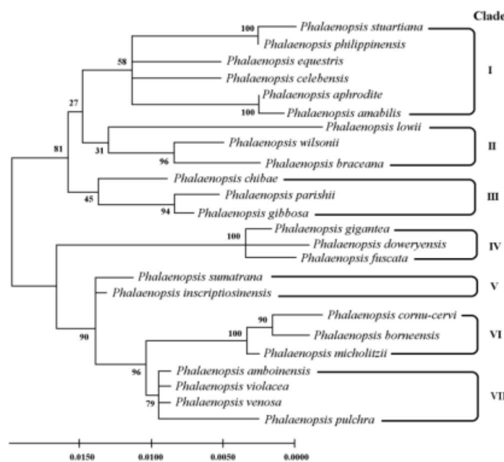

Figure 5. Phylogenetic relationship of *Phalaenopsis* based on a combined sequence of the *rbc*L and *trn*L-F. Values on the internal nodes of phylogram indicate a bootstrap analysis with 1,000 replicates

Following the bootstrap analysis, the *trn*L-F has a higher resolution of phylogenetic tree (82.35%) than the *rbc*L (60.00%). Whereas the combined sequence produces a relatively high similar resolution to *trn*L-F (80.00%). According to Nelson (2008), bootstrapping is a numerical method in generating confidence intervals that use either resampled or simulated data to estimate the sampling distribution of the maximum likelihood parameter probabilities. Hence, the *trn*L-F and the combined sequence can be useful to identify or differentiate *Phalaenopsis*, particularly at the genus level.

In general, this grouping usually corresponds to the morphological or other characteristics of each species have. For example, *P. amabilis* and *P. aphrodite* belong to the similar group based on all sequences (Table 3), presumably because they have almost the similar flower morphology (Tsai et al., 2015). Tsai et al. (2015) even included the two into one subgenus, namely *P. amabilis* complex.

At the end of the discussion, although such studies have been carried out comprehensively by several researchers, especially by Tsai et al. (2010) and Zhou (2015), we tried to combine the data from both, then deepen by determining the genetic diversity and mutations that occur therein, as well reconstructed its relationship with a simpler manner. Therefore, this information has good implications and is essential for species conservation and plant breeding programs in the future (Flint-Garcia, 2013). In other words, the results of our study have beneficial impacts, particularly for the development of new *Phalaenopsis* orchids with desirable traits.

Table 3. Grouping of *Phalaenopsis* based on the *rbc*L, *trn*L-F, and combined sequences

No	Species	Clade		
		*rbc*L	*trn*L-F	Combined
1	*P. inscriptiosinensis*	I	V	V
2	*P. sumatrana*	I	V	V
3	*P. borneensis*	I	VI	VI
4	*P. cornu-cervi*	I	VI	VI
5	*P. micholitzii*	I	VI	VI
6	*P. amboinensis*	I	VI	VII
7	*P. venosa*	I	VI	VII
8	*P. violacea*	I	VI	VII
9	*P. pulchra*	I	VI	VII
10	*P. celebensis*	II	I	I
11	*P. amabilis*	II	I	I
12	*P. aphrodite*	II	I	I
13	*P. equestris*	II	I	I
14	*P. philippinensis*	II	I	I
15	*P. stuartiana*	II	I	I
16	***P. lowii*****	III	II	II
17	*P. braceana*	III	III	II
18	*P. wilsonii*	III	III	II
19	*P. chibae*	IV	II	III
20	*P. gibbosa*	IV	II	III
21	*P. parishii*	IV	II	III
22	*P. doweryensis*	V	IV	IV
23	*P.fuscata*	V	IV	IV
24	*P. gigantea*	V	IV	IV
Average of bootstrap value** (%)		60.00	82.35	80.00

Note. *inconsistant in grouping; ** above the value of 50

CONCLUSION

Based on the *rbc*L, *trn*L-F, and their combined sequence, *Phalaenopsis* has a low genetic (nucleotide) diversity. However, this germplasm shows a complex relationship. In general, *Phalaenopsis* separated into different clades, i.e., five, six, and seven clades for each marker used, respectively. The bootstrap analysis revealed that the *trn*L-F and a combined sequence provide a high resolution of phylogenetic trees. In this case, *P. amboinensis* vs. *P. venosa* is the closest, and three other pairs (*P. gibbosa* vs. *P. doweryensis*; *P. stuartiana* vs. *P. micholitzii*; and *P. celebensis* vs. *P. pulchra*) are the farthest. Hence, both sequences can be applied to identify or differentiate *Phalaenopsis*, particularly at the genus level. The information is essential in supporting the conservation and breeding programs of *Phalaenopsis*, both locally and globally.

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