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Research Article

Genetic Variation of *Vanda foetida* J.J.Sm.; a Rare and Endemic Orchid in South Sumatra Based on RAPD Analysis

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Abstract

Vanda foetida J.J.Sm., which is an endemic orchid species in Sumatra has taxonomically disappeared for over 100 years since it was described in 1906. The distribution of *V. foetida* was limited due to over-exploitation of this orchid and changes to land-use. The study of genetic variation of *V. foetida* in its natural habitat, Mount Dempo and Padiampe, South Sumatra was conducted based on Random Amplified Polymorphic DNA (RAPD) analysis for genetic variation using 8 primers (OPU 3, OPU 5, OPU 6, OPU 7, OPU 12, OPU 13, OPU 14 and OPU 16). Genetic variation in each population was detected by h value (*Nei's genetic diversity*) which was 0.1999 and 0.1778 for Mount Dempo and Padiampe, respectively. This value was higher compared to those of other rare orchid species even though it only has a small population. *V. foetida* originated from two populations forming two main clusters in dendrogram with 67 % (0.67) degree of similarity. The dendrogram indicated that the two populations are connected, and assumed as one large population in the past and were separated from larger population by habitat fragmentation. This species was not genetically in danger and will be able to survive if its natural habitats are remained.

Keywords: orchid, *Vanda foetida*, genetic variation, RAPD, conservation.

Introduction

Vanda foetida J.J.Sm., based on its first description in 1906, has a very unique and white-purple flower. It was described as the mixing of *V. dearei* (sepal and petal was widely opened) and *V. tricolor* (which has bilobed and midlobe down-recurved labellum). The flower has an unpleasant smell, thus this orchid has the species name "foetida", which means unpleasant smell. Since described in 1906, this species has not been seen in its habitat and the world's orchid collections (Comber, 2001; Hartini & Puspitaningtyas, 2005).

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Furthermore, the holotype of *V. foetida* (BO) was only a dry herbarium of two bunches of flowers and a leaf and wet collection of a leaf and two bunches of flowers. The isotype (L) is only a bunch of dry flowers. Thus, it is assumed this species was scientifically lost for over 100 years, being rare and endemic (Metusala et al., 2010).

The population of *V. foetida* is very limited, and can only be found in Pagar Alam, South Sumatra province. This species is distributed only in small, fragmented and isolated populations in remnant tropical forests in South Sumatra. The populations are fragmented and isolated by tea and coffee plantations. Based on ecological research, the decreasing population was accelerated by deforestation, habitat fragmentation and overexploitation. In the Mount Dempo population, *V. foetida* was threatened by tea plantations, while in Padiampe, this species is threatened by coffee plantations as these estates are routinely regenerated about every 25 years. Based on interviews with locals, orchids that grow in coffee trees are highly threatened as farmers view epiphyte orchids as a parasite (Metusala et al., 2010).

Genetic structure is influenced by mating systems, effective population size, mutation rates and gene flow among populations (Raymond et al., 2003). Genetic variation appears in morphological characters of individuals such as size, colour and shape of flower and leaf as the consequences of different genetic structure among or within populations. The variations stem from different structure of DNA and even protein produced by the individual. Genetic variation is the response of the individual adapting in changed conditions of the environment (Frankham et al., 2002). Environmental changes become a factor that forced genetic variation to lead to adaptation mechanisms for those changes. Nowadays, changes in the environment are mostly caused by change in land use and deforestation. These have led to decrease, fragmentation and smaller habitats for plant populations. Habitat degradation and fragmentation yielded small patches of habitat which automatically impair population size. This may lead to limitation of pollen, reproduction connectivity and gene flow (Andrianoelina et al., 2009; Lander et al., 2010; Vranckx et al., 2012).

In small populations, gene flow is considered to be lower due to geographic isolation (Fischer et al., 2000; Parab & Krishnan, 2008). Geographic isolation consequently increases inbreeding of individuals within a population and the ability of reproduction then becomes very low. A species with restricted distribution and very small population generally maintains lower level of

genetic variation compared to common species (Chung, 2009; Li et al., 2006). On the other hand, the high fitness of species and heterozigosity support the occurrence of species in its habitat because it in turn supports the ability to adapt to environmental changes and diseases (Allendorf & Luikart, 2007). Thus, genetic variation maintenance was the main focus of plant conservation because in the long term, it affects plant fitness (Fischer et al., 2000; Frankham et al., 2002; Sun & Wong, 2001).

Genetic variation can be measured by either molecular or quantitative methods. RAPD is a fingerprinting method using short, random, oligonucleotide primers to search for variation in the entire genomic DNA. Hence, this technique is easy, allows for rapid analysis and relatively low cost. It is available to a large number of primers and requires a very small amount of DNA for analysis (Goh et al., 2005; Kishor & Sharma, 2009; Kishor & Devi, 2009; Shin et al. 2002; Ferreira et al., 2006). According to Li & Jin (2006), RAPD has superiority in detecting variation of genome compared with allozyme and can reveal greater genetic divergence between populations. This method is appropriate for study of rare species and successfully showed the relationship between population size and genetic variation.

Genetic variation among individuals and between two populations of *V. foetida* in South Sumatera is the main object to be investigated, that it may reveal adequate data of gene flow limitation between two populations, as the mating system only occurs between individuals within the population (inbreeding). The two populations of *V. foetida* from Dempo and Padiampe are separated by many kilometers in Pagar Alam city (Figure 1). To obtain information about

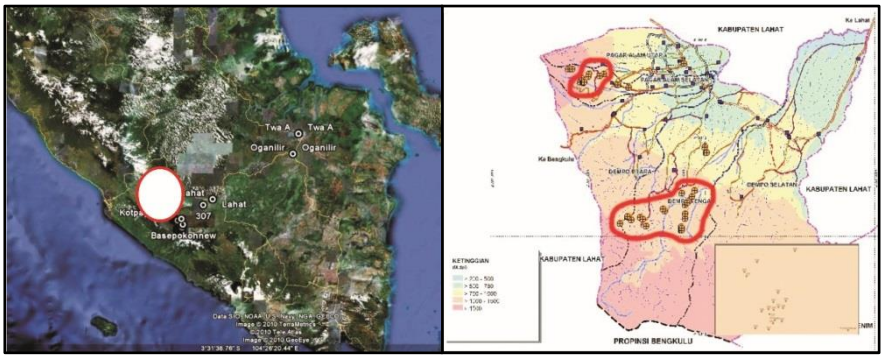

Figure 1. Location of *Vanda foetida* population in Sumatra and details of two separate populations in Pagar Alam, South Sumatra

population of *V. foetida* for its conservation, we studied RAPD profiles of two populations then addressed the questions, (1) Is there less genetic variation of *V. foetida* among individuals within each population that reveals inbreeding depression? (2) Are both populations still connected to each other?

Materials and Methods

Study of Species

The first rediscovery of *V. foetida* in its natural habitat occurred in 2008, by identification of the plant and its flower. *V. foetida* was discovered growing at unproductive rubber tree plantations. This orchid has been growing as an epiphyte at old rubber trees, and this species was destroyed by people who saw it as a weed. The rate of forest conversion and palm tree rejuvenation became a serious threat for conservation of this orchid. A recent investigation in 2010 rediscovered this species to be distributed only in small, fragmented and isolated populations in a tropical forest in Pagar Alam, South Sumatra. Only two populations of *V. foetida* were found in this area. In fragmented forests, this species was suggested as having high occurrence of inbreeding.

Study site

The two population of *V. foetida* were found in Pagar Alam, South Sumatra Province. Pagar Alam is bounded by Bengkulu Province to the south, the district of Kota Agung to the east, the district of Jarai to the north and the district of Tanjung Sakti to the west. These two populations are from the population of Mount Dempo and Padiampe. The population of Mount Dempo is located in Mount Dempo at coordinates from S4.02485 E103.15432 until S4.02912 E103.18136 and altitude at 1186-2019 metres above sea level. Most of the area in Mount Dempo was converted into tea plantations belonging to a government company, and has remnants of several small patches of original habitats near watersheds and rivers. The population of Padiampe is located in Padiampe Mountain and located at coordinates from S4.15115 E103.21304 until S4.16205 E103.24909 at 1290-1375 metres above sea level. Most areas in Padiampe were converted into coffee plantations belonging to local people.

RAPD-PCR Analysis

Ten plants of each population were used in this research. DNA isolation was successfully established using commercial DNA isolation kit Illustra Phytopure RPN-8511. Orchid samples and its code are presented in Table 1. Young leaves were cut, approximately 50-100 mg of specimen were used as the source of DNA extraction. The procedure of isolation followed the modified Tepnel Life

Sciences Company's procedure. The extracted DNA concentration and purity were quantitatively tested by GeneQuant (spectrophotometer) with wave length of 260 and 280 nm. The DNA and primers were diluted to obtain the appropriate and optimum concentration for PCR procedure, which was 25 ρM for primers. The PCR-RAPD profile of samples were performed by PCR *mix* Go Taq® Green (Promega) using 8 specific primers successfully used for *Vanda* (Orchidaceae), i.e OPU-3, OPU-5, OPU-6, OPU-7, OPU-12, OPU-13, OPU-14 and OPU-16 (Lim et al., 1998). Horizontal agarose gel electrophoresis apparatus (BioRad electrophorator) was used to run DNA through gels. Visual profiles of band in electrophoresis gels were printed and the absence and presence of band in every locus from all orchid samples were scored.

Table 1. Sample code of *V. foetida*

No.	Sample Code	Population
1	139	Dempo
2	275	Dempo
3	276	Dempo
4	278	Dempo
5	279	Dempo
6	283	Dempo
7	285	Dempo
8	290	Dempo
9	299	Dempo
10	22.11	Dempo
11	314	Padiampe
12	350	Padiampe
13	367	Padiampe
14	368	Padiampe
15	369	Padiampe
16	372	Padiampe
17	373	Padiampe
18	384	Padiampe
19	385	Padiampe
20	397	Padiampe

Data Analysis

The percentages of polymorphic locus for each primer were counted in this study. The percentages of polymorphic locus in each population were analyzed using POPGEN 1.32 (Yeh et al., 1999). Genetic variation of each population (h-value) was analyzed using the POPGENE version 1.32 programme (Yeh et al., 1999) while genetic variation between populations was analyzed through the Unweighted Pair-group Method Using Arithmetic Average (UPGMA) (Sneath & Sokal, 1963). A pPhylogeny tree was constructed and its data was carried out in NTSYSpc21. The binner data was used to construct scatter plots using the GenAlEx version 6.1 programme to show molecularly position of all samples in the population.

Results and Discussion

DNA Amplification result

Amplification result of total genom in 20 samples of *V. foetida* using 8 primers revealed that there were 117 DNA fragments from 150 – 3000 bp. Among these, 107 fragments were polymorphic (91.45 %) and only 10 fragments were monomorphic (8.55 %). Bands of all orchid samples using OPU 12 primer is shown in Figure 2.

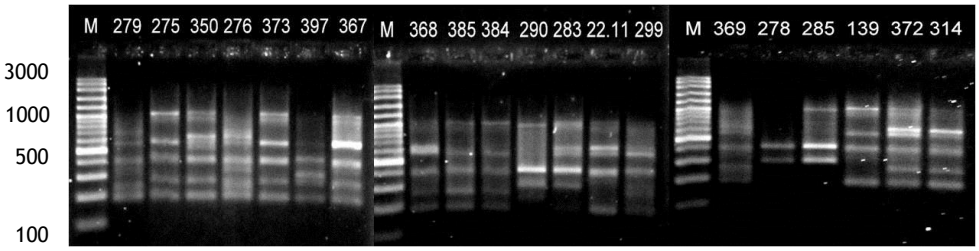

Figure 2. Amplification result of *V. foetida* using OPU 12 primer. M = DNA marker (Vivantis 100 bp), numbers are showing sample code

Tabel 2. Details of 10-mer random primers screened for this study and degree of polymorphism and information content for 8 RAPD random primers along with their sequences, applied to 20 samples of *V. foetida*

Primer	Nucleotide Sequence (5'-3')	Amplified Locus	Monomorphic Locus	Polymorphic Locus	(%)
OPU 3	CTATGCCGAC	16	0	16	100
OPU 5	TTGGCGGCCT	13	1	12	92.31
OPU 6	ACCTTTGCGG	15	0	15	100
OPU 7	CCTGCTCATC	17	0	17	100
OPU 12	TCACCAGCCA	14	1	13	92.86
OPU 13	TGGGTCCCTC	12	0	12	100
OPU 14	CTGCGCTGGA	14	1	13	92.86
OPU 16	GGCTGGTTCC	16	7	9	56.25
	Total	117	10	107	91.45%

Based on the percentage of polymorphic loci in each primer (Table 2), it shows that in general, 8 primers can be used in RAPD method for *V. foetida* because it produced a fairly high polymorphism (above 50 %). The percentage of polymorphic loci of 100 % was generated through the OPU-3 primer, OPU-6, OPU-7 and OPU-13. This indicated that four primers can amplify loci on the genome of *V. foetida* more than other primers used in this study. Moreover,

the primers can also amplify some specific fragments in the sample of *V. foetida*, so that the primer was optimal for this species

Genetic variation

In this study, the analysis of polymorphic percentage was established. This analysis aimed to identify the difference in polymorphism level in each population of *V. foetida* which revealed genetic variation. In this study, 117 loci were successfully amplified from eight primers. The percentage of polymorphic loci analysis results are presented in Table 3.

Table 3. Percentage of polymorphic locus in each population

Population	Polimorphic Locus	Polimorphic Locus Percentage
Dempo	89	76.07 %
Padiampe	83	70.94 %

The number and percentage of polymorphic loci in each population showed the presence of more than one allele in a locus. Based on the above data, it showed that both populations have a fairly high polymorphism, above 70 %. The existence of polymorphic loci led to the presence of genetic variations (Chung, 2009; Li et al., 2002). The high level of polymorphic loci was also reported in a study of the genetic relationship between species in *Phalaenopsis*, which were between 26-54 polymorphic loci for each primer (Niknejad et al., 2009).

Polymorphism in populations of *V. foetida* may occur, due to random mating in the population. *Vanda* is compatible to the pollen of other orchid genus. However, genus of *Vanda* evolved specifically to form the pollen and its pollinator becomes very specific (Motes, 1997). This mechanism occurs because of orchid pollen called polinaria. Polinaria causes pollen to stick well on pollinators so that pollen can be carried over to another pistil of different trees (Arditti, 1992; Cozzolino & Widmer, 2005). Furthermore, random mating in a population of cross-pollinated plants will tend to maintain heterozygosity in nature (Frankham et al., 2002).

In *V. foetida*, cross-pollination occurs between individuals in similar population. This occurs because two populations of *V. foetida* (Mount Dempo and Padiampe) were separated by an extensive urban area, Pagar Alam City. With the considerable barrier between the two populations, pollination can only occur between individuals within a population, without any cross-pollination between the two populations. Therefore, there is no gene flow

between the two populations. If each population lost most of its members as a result of habitat exploitation, both populations will experience inbreeding depression due to reduced random mating events. Inbreeding is an event which produces offspring of close related individuals. Inbreeding can decrease the level of reproduction, survival, and power successor descent. This leads to offspring which have high risk of death (Allendorf & Luikart, 2007). It could threaten the survival of the population.

Genetic variation within and between populations

Genetic variation in each population was detected by h value (*Nei's genetic diversity*) which was 0.1999 and 0.1778 for Mount Dempo and Padiampe, respectively (Table 4). According to Chung (2009), genetic variation within the population of *V. foetida* was higher than those of some rare species originating from South Korea, *Gymnadenia cucullata*, *Gymnadenia camtschatica*, *Amitostigma gracile*, and *Pogonia minor*. H value of their population is only 0.036, 0.067, 0.009 and 0.014, respectively. This result showed that each population of *V. foetida* still had high genetic diversity. The existence of genetic variation showed that the population of *V. foetida* was not in danger of extinction even though the population was small. The information of genetic variation can be used to support *V. foetida* conservation, which mainly focuses on increasing the number of individuals and also enriching genetic variation to maintain the existence of *V. foetida* populations.

Table 4. The value of h (*Nei's genetic diversity*)

Population	h (*gene diversity*)
Dempo	0.1999 ± 0.1673
Padiampe	0.1778 ± 0.1714

Based on genetic variation, it was known that genetic variation in the population of Mount Dempo was higher than those of Padiampe. This happens with regards to habitat conditions of both populations. At Mount Dempo, most of the area which was originally a forest has been converted into tea plantations. The high genetic variation in the population of Mount Dempo indicated that initially this population was a huge and stable and genetic variation was well maintained. Genetic variation of *V. foetida* in Mount Dempo was maintained by the residents by growing this species in their garden (Metusala et al., 2010). Padiampe population is a natural population located in protected forest area. However, this protected forest has changed as a result of coffee-growing by local communities. Then, some species of host plants for

V. foetida were replaced by coffee trees. The alteration of host plants may cause genetic changes that triggered the onset of genetic variation in the Padiampe population. Genetic variation in both populations of *V. foetida* was a result of cross-pollination that occurs between individuals within a population.

The diversity between populations of *V. foetida* was analyzed using NTSYSpc21 expressed by genetic similarity coefficient (Rohlf, 1997). Similarity coefficient indicates the extent of genetic similarity among members of a population so that it can be used to formulate individual clusters by using UPGMA. Individual clusters show the diversity between populations (Singh, 1999). Analysis using NTSYSpc21 formed dendrogram presented in Figure 3.

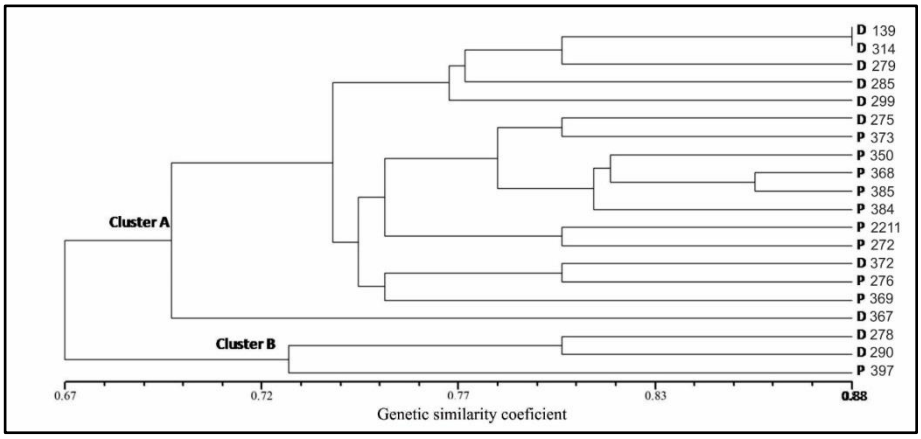

Figure 3. Dendrogram of *V. foetida* from two populations, population of Dempo (D) and Padiampe (P). Individual samples of *V. foetida* were identified using numeric code as it was collected in Purwodadi Botanic Garden, East Java, Indonesia. Numeric codes of populations correspond to those in Table 1

In addition, description of population composition based genetic analysis was established using the GenAlEx 6.1 programme (Peakall & Smouse, 2005). Scatter plot showed the distribution of the individuals in population of *V. foetida* based on molecular character of RAPD analysis. Scatter plot is presented in Figure 4. Based on RAPD analysis for genetic variation using the 8 primers, it showed that *V. foetida* which originated from two populations formed two main clusters in dendrogram with 67 % (0.67) degree of similarity. The two populations of *V. foetida* were closely related. This phenomenon was also exhibited by the grouping of individuals from both populations into one cluster in dendrogram with 74 % (0.74) degree of similarity. Dendrogram (Figure 3) revealed that individuals formed two large clusters, namely A and B.

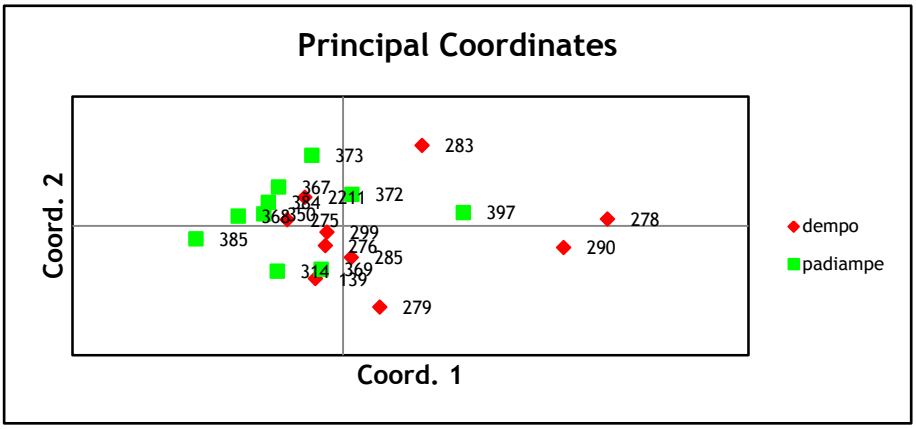

Figure 4. Scatter plot of RAPD analysis of *V. Foetida* in two populations. Red dots indicated individuals from population of Mount Dempo. Green dots indicated individuals from population of Padiampe

In cluster A, most individuals of *V. foetida*, from both populations of Mount Dempo and Padiampe, clumped and mingle. These individuals are clustered at the level of similarity 72 % (0.72). This is different from the concept of similarity by Singh (1999) which states that individuals with morphological similarity up to 85 % can be suggested as the same species. The level of similarity in this study refers to the phenetic molecular basis character, so that the level of similarity in one species can have different values from the concept of morphological similarity.

Based on the dendrogram and scatter plot, it is known that there are some individuals withlower levels of similarity compared to other individuals. Individuals with code number 283 were contained in cluster A, and individuals with sample code 397, 278, and 290 were contained in cluster B. Individuals with sample code 397 came from Padiampe, while individuals 278, 290, and 283 were from the population of Mount Dempo. The individual of 283 were grouped in clusters A at the level of similarity 70 % (0.70), while the individuals of 397, 278, and 290 were in large clusters which have level of similarity 67 % (0.67) with clusters A. The individual distribution on scatter plot in Figure 4 also showed that individuals with the sample code 283 seemed a bit detached with another individual of cluster A, while individuals which come from cluster B did not seem to be clustered with members of cluster A. This showed that the two populations revealed genetic variation in the populations.

Individuals from population of Mount Dempo which are 276, 283, 290, 278 and 385 clustered with individuals in cluster B in the level of similarity 67 %. This clustering showed that *V. foetida* population which was currently divided into two populations are still connected and assumed as one large population in the past. This large population was then separated by habitat fragmentation due to the development of human urban civilization which became a barrier for both populations. This was supported by the appearance of the estimated population distribution of *V. foetida* on a scatter plot (Figure 4). From the scatter plot, we know that most of individuals from Mount Dempo and Padiampe populations are clustered. So, it was clear that the two populations were in the beginning one large population.

Habitat fragmentation also occurred in populations of the ground orchid *Pterostylis gibbosa* from Australia. Genetic variation in populations of *P. gibbosa* is indicated by the percentage of polymorphic loci 69 % and heterozygosity (h) 0.261 (Sharma et al., 1999). This orchid population showed high genetic variation. Small population as the result of habitat fragmentation still can have high genetic variation, yet it is needs to be sustainably maintained for its conservation (Li & Ge, 2006). In populations of *V. foetida*, the possibility of natural gene flow through cross-pollination between the two populations is very low because both are constrained by geographic barriers. But, the high genetic variation can compensate the negative impact of habitat fragmentation which reveals the capability of adaptation to a changing environment. This species can have stable population with huge population size and high genetic diversity if the number of individuals increase and cross-pollination between the two populations occurs. *Ex-situ* conservation strategy should be established to increase population size and genetic diversity using artificial cross-pollination between the two populations. Therefore, introduction of its offspring into the natural habitat will able to maintain its existence in nature (Chung, 2009; Young et al., 1996).

Conclusion

We can conclude that the two populations of *V. foetida* which are Mount Dempo and Padiampe, Pagar Alam, South Sumatra have high genetic variation even though this species only survives in small populations. This species is not genetically in danger and will able to survive if its natural habitats are remained. The two populations are genetically connected and are assumed as one large population in the beginning.

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