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ANALYSIS OF GENETIC DIVERSITY AMONGST POPULATIONS OF *VANDA THWAITESII* HOOK.F. AND *VANDA WIGHTII* RCHB.F., TWO NOTIFIED ENDANGERED ORCHIDS OF WESTERN GHATS, INDIA

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Abstract

Understanding of genetic diversity is very important for the management of conservation in any endangered species. *Vanda thwaitesii* Hook.f. and *V. wightii* Rchb.f. are two notified endangered orchids of Western Ghats, India demanding evaluation of genetic diversity for conservation action and thus presently, these studies have been carried out. ISSR profile of 20 samples from 15 populations of *Vanda thwaitesii* in a spatial separation of 10-286 km from type locality exhibited very low heterozygosity (h=0.083) and reduced gene flow (Nm=0.01). The populations clustered into 3-4 groups without any correlation between geographic locations of plant distribution. Twenty eight samples of *V. wightii* from 15 distinct populations separated by 40-264 km from type locality revealed moderate diversity (h=0.25; Nm=0.98) but not having correlation with respect to geographical separation. Occurrence of majority of populations in highly fragmented habitats, low levels of genetic diversity and reduced/negligible gene flow shows the immediate requirement of conserving *V. thwaitesii*. In spite of the existence of moderate diversity, distribution of diversity amongst the populations in disturbed forests and inhabited land necessitate the rehabilitation/reinforcement of *V. wightii* into protected forests.

Introduction

THE EXISTENCE of biodiversity has prime importance for the stability of an ecosystem and thus developing effective strategies for their conservation is the serious concern of conservation biologists (Singh *et al.,* 2017). Understanding of the genetic diversity in a species is also recognized as very crucial for conservation action. The genetic diversity of any species may appear spatially structured at different scales, such as population, sub-population or among neighboring individuals (Escudero *et al*., 2003; Thomas *et al*., 2020) and can provide important information on the levels of genetic variation and the partitioning of this variability within/between populations is important for conservation planning (Ellis and Burke, 2007). Knowledge of the genetic diversity can also provide vital information for plant improvement (Li *et al*., 2014a).

Vanda thwaitesii Hook.f. and *Vanda wightii* Rchb.f. are two vandas amongst the 84 species belonging to the genus reported worldwide (POWO, 2021). These are endemic to Southern Western Ghats, India and Sri Lanka (Sathish Kumar and Suresh Kumar, 1998; Sathish Kumar *et al.,* 2006) and are endangered mainly due to habitat loss and fragmentation. Even though *V. thwaitesii* was first collected by Thwaites from Sri Lanka in 1898, it remained elusive for over a century which forced to declare the species as extinct, in 1981 (Sathish Kumar and Suresh Kumar, 1998). However, the species was collected from Silent Valley and Wayanad, Kerala during 1982 to 1997 (Sathish Kumar and Suresh Kumar, 1998) and later reported from Periyar Tiger Reserve and Nelliyampathy in Kerala; Coorg, Hassan, and Chikmagalur districts of Karnataka; and Nilgiri District of Tamil Nadu (Augustine, 1995; Kumar, 2016; Sankara *et al*., 2019; Sathish Kumar and Suresh Kumar, 1998; Sharlef and Murthy, 2011; Sreekumar *et al*., 2017). *Vanda wightii* Rchb.f. is originally described from Vaulyar and Palghatcherry in 1849 and Thwaite's collection from Sri Lanka (Sathish Kumar *et al.,* 2006) and is supposed to be extinct as it has not been re-sighted in the wild, ever since the type collection (Limansela *et al.,* 2002). However, during 2000-02 period, the species was re-collected from Dakshina Kannada district of Karnataka; Kannur and Palakkad district of Kerala (Sathish Kumar *et al.,* 2006). Both the species are described to have narrow distribution with restricted numbers and later under section 38 of the Biological Diversity Act 2002, the Central Government notified both the species to prohibit/ regulate collection (MOEF, 2009) and invited various studies on all aspects of the species for holistic understanding and propagation for the purpose for *in situ* and *ex situ* conservation. As proved effective in a wide range of plant species including orchids as *Calanthe* (Qian *et al*., 2013), *Cattleya* (Rodrigues *et al.,* 2015), *Cymbidium* (Li *et al*., 2014; Sembi *et al*., 2020), *Dendrobium* (Bhowmik and Rahman, 2020; Feng *et al*., 2013; Gurudeva, 2019), *Oeceoclades maculata* (Ueno

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et al., 2015), and *Vanda* (Lekshmi and Decruse, 2018; Madhavi and Shankar, 2019; Manners *et al*., 2013; Sunita *et al*., 2021), we used inter simple sequence repeat (ISSR) markers (Wolfe *et al*., 1998), to understand the genetic diversity and structuring of natural populations of *V. wightii* and *V. thwaitesii* from Western Ghats region of Kerala, Tamil Nadu, and Karnataka. Information on the genetic diversity and structure of both the species is lacking and this is the first study using molecular markers for these species so as to characterize genetic diversity of their populations.

Material and Methods

Sampling

Populations of *V. thwaitesii* have distribution in Western Ghats region of Kerala including Wayanad, Silent Valley, and Periyar Tiger Reserve (Augustine, 1995; Sathish Kumar and Suresh Kumar, 1998) and Coorg to Chikmagalur district in Karnataka (Kumar, 2016; Sankara *et al*., 2019; Sharlef and Murthy, 2011). Both the species have distribution at altitudes 500 to 1060 m in moist deciduous to evergreen forests. *Vanda wightii* has fragmented populations existing mainly in Dakshina Kannada, Kasargod, Kannur, Malappuram, Palakkad, and Idukki districts in Karnataka and Kerala at 34-1023 m altitudes in deciduous forests (Decruse, 2014; Sathish Kumar *et al.,* 2006). Adult plants of *V. thwaitesii* were collected from the reported localities from Idukki district of Kerala to Coorg district of Karnataka (Fig. 1) and *V. wightii* collected from Idukki to Kasaragod districts of Kerala (Fig. 1); these were maintained at field conservatory of JNTBGRI and subjected to genetic diversity analysis.

Genomic DNA Isolation, ISSR Amplification, and Data Analysis

A total of 20 plant samples of *V. thwaitesii* from 15 populations spread over Idukki district of Kerala to Coorg district of Karnataka with a total map distance of about 366 km (Table 1; Fig. 1) and 28 samples of *V. wightii* from 15 populations spread over Idukki to Kasaragod district of Kerala (Table 2; Fig. 1) were subjected to genetic diversity analysis using ISSR profiling (Vijayan and Chatterjee, 2003). Young leaves weighing about 500 mg were collected from each mother plant and the genomic DNA extracted as per the CTAB method described elsewhere (Anto *et al.,* 2020; Murray and Thomson, 1980). The isolated DNA samples obtained

Fig. 1. Map showing populations of *Vanda wightii* (thick line) and *Vanda thwaitesii* (Thin line) subjected to genetic diversity analysis.

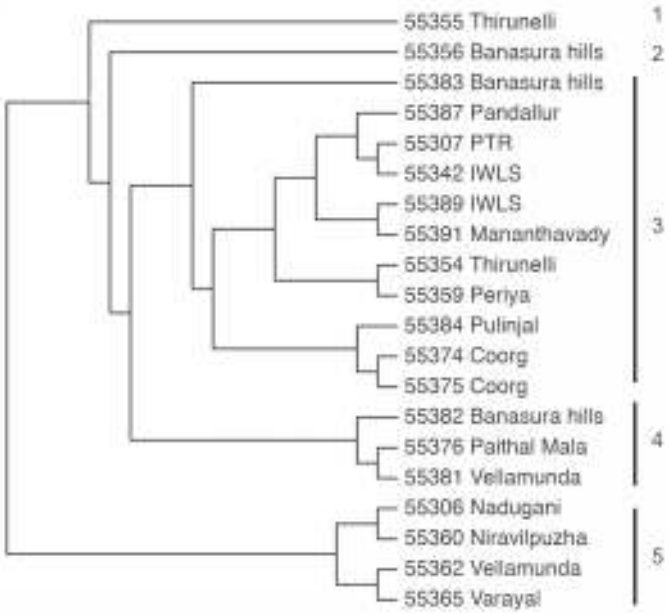

Fig. 2. Dendrogram based on the ISSR products derived from 20 samples of *V. thwaitesii* from 15 populations. PTR, Periyar Tiger Reserve; IWLS, Idukki Wildlife Sanctuary.

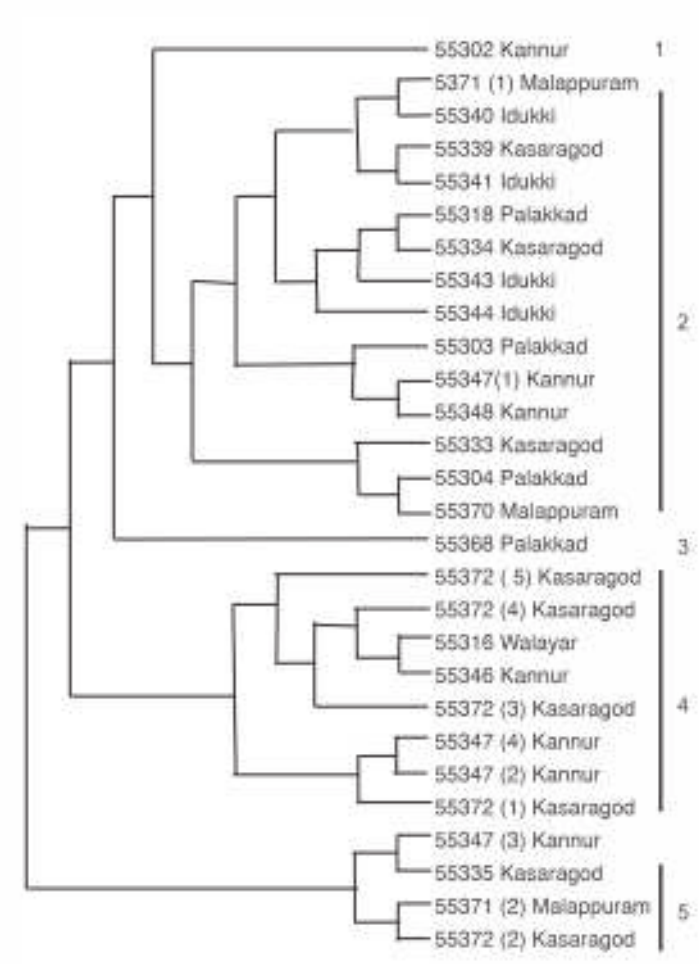

Fig. 3. Dendrogram based on the ISSR products derived from 28 samples of *V. wightii* from 15 populations.

after ethanol precipitation were re-suspended in 100 µl 1X TE and stored at 20°C for future analysis. The isolated genomic DNA was used as template for ISSR assays which was carried out with 10 primers of 17-18 bp for *V. thwaitesii* and 15 primers of 17-18 bp for *V. thwaitesii* (Table 3). The PCR reaction mixture was prepared to 25 µl volume containing 50 ng of DNA template, 0.2 mM dNTP mix, 1X reaction buffer, 20 pmol primer, and 1 unit Taq DNA Polymerase (Finnzymes, India) and double distilled water. The reaction mixture concentration and PCR conditions were standardized by trials. The amplification was performed using Eppendorf Thermocycler with a hot start at 94°C for 2 min; followed by denaturing at 94°C for 15 sec of 35 cycles each; annealing for 15 sec at 37°C; and product extension time for 5 min at 72°C. The amplified products were resolved in agarose gel (1.5%) containing Ethidium Bromide in a submarine electrophoresis unit (BioRad Inc.) and visualized under Gel documentation system (Almeida-Pereira *et al.,* 2017). The products were scored and used for assaying various genetic diversity indices. Similarity matrix was developed using the WINDIST software. The matrix thus generated was used to prepare dendrogram using the FreeTree software. Bootstrap method was followed to build a UPGMA based dendrogram. A total of 1,00,000 tree repetition count was made to build the most probable tree. Nei's gene diversity at population level (h), Shannon index (I), and expected number of alleles were calculated to estimate genetic variation levels using POPGENE program (version 1.31, Yeh *et al.*, 1999).

Results

Vanda thwaitesii

The 20 plant samples from 7-8 populations spread over Idukki district of Kerala to Coorg district of Karnataka covering a total of 334 km map distance did not show much diversity amongst populations, in ISSR profile. The 10 ISSR primers generated a total of 56 bands (3-12 per primer) of which 15 were polymorphic (Table 3). The dendrogram (Fig. 2) shows that the 20 samples are distributed in 3-4 clusters. No distinct groups in relation to either altitude or geographical regions could be identified. Different populations from Wayanad appeared in 2 separate clusters (Cluster 4 and 5; Fig. 2) and 2 outliers. However, populations from distant locations in Coorg, Nilagiri, Periyar Tiger Reserve (PTR), and Idukki Wildlife sanctuary (IWLS) with 50-286 km map distance from type locality, clustered together with a few populations from Wayanad (cluster 3; Fig. 2). The collections also showed relatively low level of genetic diversity with Nei's gene diversity (h)=0.083 and Shannon's diversity index (I)=0.13 (Table 4). The mean value of heterozygosity (Ht) observed in the various accessions of *V. thwaitesii* was 0.083 and the mean value of average heterozygosity was 0.01. Similarly, the heterozygosity values were very less (0.083) while degree of genetic differentiation (Gst) showed relatively high value 0.99 (Table 4). The other diversity measures (Table 4) also showed low values.

Vanda wightii

The 28 plant samples from 15 populations spread over Idukki to Kasaragod district of Kerala covering a total 384 km map distance showed marginal diversity amongst *V. wightii* populations as per ISSR profile. The fifteen ISSR primers gave a total of 84 bands (3-11 per primer) of which 27 were polymorphic (Table 3). The 28 samples were distributed in 3 clusters in addition to 2 collections as outliers without any distinct group in relation to geographical separation (Fig. 3). Still, the clusters showed a regional grouping especially the accessions from south extreme in Idukki district clustered together along with a few accessions from other locations with a map distance of 187-384 km. Among a few sub-populations sampled

Table 1. Populations of *Vanda thwaitesii* subjected to ISSR profiling.

Collection number	Coordinates		Location	District	Altitude (m)	Distance from type locality (km)
55391	N11.82964	E75.97631	Mananthavady*	Wayanad	732	0
55355	N11.91035	E75.98507	Thirunelli	Wayanad	792	10
55354	N11.91035	E75.98507	Thirunelli	Wayanad	792	10
55365	N11.83887	E75.91282	Varayal	Wayanad	722	12
55362	N11.73577	E75.92968	Vellamunda	Wayanad	745	14
55381	N11.73588	E75.93034	Vellamunda	Wayanad	742	14
55384	N11.71252	E75.94255	Pulinjal	Wayanad	752	15
55382	N11.71853	E75.92245	Banasura hills	Wayanad	930	16
55383	N11.6949	E75.93852	Banasura hills	Wayanad	852	17
55359	N11.8426	E75.86883	Periya	Wayanad	827	17
55356	N11.71355	E75.91922	Banasura hills	Wayanad	1001	17
55360	N11.73865	E75.83483	Niravilpuzha	Wayanad	742	23
55387	N11.49433	E76.33644	Pandallur	Nilagiri	992	50
55387	N11.49433	E76.33644	Pandallur	Nilagiri	992	50
55306	N11.46208	E76.4135	Nadugani	Nilagiri	881	58
55376	N12.15478	E75.56917	Paithalmala	Kannur	489	61
55374	N12.3545	E75.65257	Chettimani	Coorg	901	72
55375	N12.37643	E75.56927	Cherambane	Coorg	885	78
55389	N9.749731	E76.97291	IWLS	Idukki	980	256
55307	N9.482617	E77.14473	PTR	Idukki	910	286

IWLS, Idukki Wildlife sanctuary; PTR, Periyar Tiger Reserve; *One type locality.

from Kannur (55347, Srikantapuram) and Kasaragod (55372, Adhur-pandy), the accessions appeared in the same or nearest cluster. Besides, accessions from different locations also appeared in the same cluster (clusters 2, 4, 5; Fig. 3). The collections showed low level of genetic diversity with Nei's gene diversity (h)=0.25 and Shannon's diversity index (I)=0.39. The mean value of heterozygosity (Ht) observed in the various accessions of *V. wightii* was 0.25 and the mean value of average heterozygosity was 0.17. The accessions showed appreciable Gene flow (Nm) with the value 0.98 (Table 4).

Discussion

Conservation planning, action monitoring, and evaluation of a species initially require prioritization through threat assessment and primary data on their genetic structure (Mace and Lande 1991; Master, 1991; Moran and Kanemoto, 2017). *Vanda thwaitesii* and *V. wightii* are two epiphytic orchids endemic to Indian Peninsula and Sri Lanka unknown for over 100 years (Sathish Kumar and Suresh Kumar, 1998; Sathish Kumar *et al.,* 2006) and Government of India notified these species as endangered preventing further loss. Studies on holistic understanding and propagation of these notified species need to be carried out for conservation purposes. Thus, as part of a sponsored project supported by DBT, Government of India, detailed study has been undertaken to analyze the genetic diversity through ISSR profiling for conservation implication.

The distribution of both the species is distinct with altitudinal preferences. *Vanda thwaitesii* prefers sub-tropical habitats but *V. wightii* prefers tropical habitats. However, both have common occurrence in Idukki wildlife sanctuary, and still having altitudinal separation.

Table 2. Populations of *Vanda wightii* subjected to ISSR profiling.

Collection number	Coordinates		Location	District	Altitude (m)	Distance from type locality (km)
55339	N9.77721	E77.06794	Anchuruli	Idukki	870	120
55343	N9.7716	E77.07495	Anchuruli	Idukki	768	120
55340	N9.75965	E76.98552	Kizhukkanam	Idukki	830	119
55341	N9.75319	E76.98264	Kizhukkanam	Idukki	813	119
55344	N9.827	E77.01352	Kalvary mount	Idukki	1023	112
55303	N10.9941	E76.40357	Kottoppadam	Palakkad	95	50
55304	N10.9942	E76.40362	Kottoppadam	Palakkad	95	50
55368	N10.9520	E76.40685	Meppara	Palakkad	94	48
55318	N10.5366	E76.62093	Pothundy	Palakkad	198	40
55316	N10.8262	E76.81771	Walayar*	Palakkad	176	0
55371(1)	N11.0770	E76.26683	Melattur	Malappuram	58	67
55371(2)	N11.0770	E76.26683	Melattur	Malappuram	58	67
55370	N11.2655	E76.20875	Vadapuram	Malappuram	34	83
55302	N12.068	E75.50135	SK Puram	Kannur	114	199
55347(1)	N12.068	E75.50122	SK Puram	Kannur	103	199
55348	N12.0682	E75.51436	SK Puram	Kannur	124	198
55346	N12.0235	E75.52561	SK Puram	Kannur	16	194
55347(3)	N12.068	E75.50122	SK Puram	Kannur	103	199
55347(4)	N12.068	E75.50122	SK Puram	Kannur	103	199
55347(2)	N12.068	E75.50122	SK Puram	Kannur	103	199
55335	N12.5377	E75.21125	Addur-Pandy	Kasargod	209	257
55372(2)	N12.5303	E75.21622	Addur-Pandy	Kasargod	168	257
55372(5)	N12.5303	E75.21622	Addur-Pandy	Kasargod	229	258
55372(4)	N12.5303	E75.21622	Addur-Pandy	Kasargod	180	258
55372(3)	N12.5303	E75.21622	Addur-Pandy	Kasargod	180	258
55372(1)	N12.5303	E75.21622	Addur-Pandy	Kasargod	229	258
55334	N12.5367	E75.16068	Poovadka	Kasargod	184	262
55333	N12.5367	E75.16068	Karadka	Kasargod	183	264

SK Puram, Srikantapuram; *Type locality.

The populations of both the species are highly fragmented. *V. thwaitesii* having a distribution extant of about 364 km map distance from Gavi in the South extreme to Cherambene in the North extreme possesses very poor genetic heterogeneity (Ht=0.083) and gene flow (Nm=0.01). It is an indication of poor genetic base and thus is highly vulnerable to genetic erosion. Exact reason for reduced heterogeneity between populations over a distance of about 364 km is not clear. However, their narrow population in restricted numbers, in highly fragmented habitats suggests the existence of inbreeding amongst their populations. The latter breeding system further narrows the genetic base and thus adds to the risk of

Table 3. List of primers and their sequence used for ISSR analysis and the output as bands obtained in gel electrophoresis.

ISSR	Sequence	*V. wightii*		*V. thwaitesii*	
		Number of bands	Polymorphic bands	Number of bands	Polymorphic bands
808	AGAGAGAGAGAGAGAGC*	4	0		
815	CTCTCTCTCTCTCTCTG*	5	1		
816	CACACACACACACACAA*	5	4		
817	CACACACACACACACAA*	5	1		
818	CACACACACACACACAG*	5	2		
829	TGTGTGTGTGTGTGTGC*	7	7		
834	AGAGAGAGAGAGAGAGYT	3	0	4	0
835	AGAGAGAGAGAGAGAGYC	8	4	12	3
836	AGAGAGAGAGAGAGAGYA	5	1	7	1
840	GAGAGAGAGAGAGAGAYT	6	0	5	0
841	GAGAGAGAGAGAGAGAYC	6	0	4	1
843	CTCTCTCTCTCTCTCTRA	4	1	3	0
844	CTCTCTCTCTCTCTCTRC	3	2	3	2
845	CTCTCTCTCTCTCTCTRG**			4	0
847	CACACACACACACACARC	11	3	6	1
848	CACACACACACACACARG	7	1	8	7
Total		84	27	56	15

* Used for *V. wightii* only; **Used for *V. thwaitesii* only.

extinction of the plant species. The existence of genetic diversity is very important for the long-term survival of a species, because loss of genetic variation within populations may significantly decrease adaptability to environmental challenges thus increasing extinction risk (Izawa *et al*., 2007). This extensively happened in *V. thwaitesii* through extensive loss of habitats, in the places with ideal climatic conditions. *Vanda wightii* populations subjected to diversity analysis possessed maximum spatial separation of about 384 km and separated by 40-264 km from type locality. Still, the populations exhibited only moderate genetic diversity (Ht=0.25) and gene flow (Nm=0.98). Habitat loss and fragmentation extensively happened to most of its populations as their habitats are mostly in middle and low land where a total loss of forest land occurred. The existence of present diversity is probably due to inter-breeding, before geographical isolation.

Generally, widespread species tend to possess higher genetic diversity than endangered and endemic species (Chen *et al*., 2014; Yu *et al*., 2011). There are also some case reports of rare or endemic species having high genetic diversity (Chen *et al.,* 2014; Gonzalez-astorga and Castillo-campos, 2004). It is proved through extensive studies on widespread, endangered or endemic species that abundant genetic variation and diversity characteristics of a species are not directly correlated to individual numbers, but inherited from their ancestors and parents, so that their seedlings have high genetic diversity at species level (Yu *et al.,* 2011). *Vanda thwaitesii* is an endemic species having populations exhibiting poor variation in floral and other morphological characters which is reflected also in the ISSR profiling so that representative samples from 20 populations clustered together without any correlation between geographic distances. Still, the Wayanad populations with 10-50 km spatial distance from type locality showed small variations hence these appeared in different clusters. Whereas, populations from other localities as Periyar Tiger Reserve (PTR), Idukki Wildlife sanctuary (IWLS), Pandallur, and Coorg shared a common cluster along with a few Wayanad populations. Therefore, the

Table 4. Genetic diversity estimates of the populations of *Vanda thwaitesii* and *V. wightii.*

Diversity indices	Values	
	V. wightii	*V. thwaitesii*
Number of assay units (Loci)	15	10
Number of products	84	56
Number of polymorphic loci	27	15
Percentage polymorphic loci	32.15%	26.79%
GS	0.95	0.93
Na (Number of Different Alleles)	1.33	1.27
Ne (Number of Effective Alleles)	1.24	1.13
H (Nei's gene diversity)	0.25	0.083
I (Shannon's Information index)	0.39	0.13
Ht	0.25	0.083
Hs	0.17	0.01
Gst	0.34	0.99
Nm	0.98	0.01

Na, observed number of alleles; Ne, effective number of alleles; H, Nei's (1973) gene diversity; I, Shannon's diversity index; Ht, observed mean heterozygosity; Hs, Mean value of average heterozygosity; Gst, genetic differentiation; and Nm, gene flow.

Wayanad population needs special attention for conservation implication. As suggested in *Calanthe tsoongiana* (Qian *et al.,* 2013), the restricted gene flow in *V. thwaitesii* might be due to habitat fragmentation and reduced population size as a result of anthropogenic activities. Still, higher number of fruits and seedlings observed in Wayanad (Unpublished information) indicates no reproductive problems and thus the environmental conditions are conducive for natural seedling recruitment. Thus the species can evolve further if the genetic diversity from the fragmented populations is reinforced into protected forests in nearby localities possessing self-sustaining populations.

Vanda wightii is also an endemic species now confined to Western Ghats, India but a moderate genetic diversity is existing among the fragmented populations as indicated by the diversity values (Table 3) and hierarchical cluster (Fig. 3). Small amount of variation in floral characters among different populations (Unpublished information) substantiate the diversity data. The populations in subtropical area at 768-1023 m altitude in Idukki district clustered along with a few populations in Kasaragod, Kannur, Malappuram, and Palakkad with 40-264 km spatial distances and 34-198 m altitudes thus showing the absence of any correlation between geographic locations of its distribution. Even though not worked out in detail, the sub-populations sampled from Kannur and Kasargod appeared in different clusters along with the samples from other localities showing intra-population variation as well.

Information on the spatial distribution of genetic diversity is very important for a better understanding of the relationships between life-history characteristics, stochastic factors, gene flow, and environmental influences (Escudero *et al*., 2003). However, there was no evidence of the occurrence of isolation by distance among the locations sampled for both *Vanda thwaitesii* and *V. wightii* which indicated greater genetic similarity between spatially distant populations. The lack of correlation between genetic and geographic distances of sampled populations is probably due to multiple events of introduction and casual dispersal events, mediated by human action as suggested in *Cirsium arvense* (Guggisberg *et al*., 2012). According to our observations, both *Vanda thwaitesii* and *V. wightii* usually occur in disturbed forests, outer margin of forests receiving ample sunlight and inhabited area but not deep inside the preserved forests. Therefore, the absence of correlation between genetic and geographic distances of both *Vanda thwaitesii* and *V. wightii* populations suggests that the spatial distribution of genetic diversity of these species seem to be influenced by their reproductive system and ancient colonization by seed dispersal through long distances by wind (Dressler, 1993).

Conclusion

Low levels of genetic diversity and reduced/negligible gene flow in different populations of *Vanda thwaitesii* show the immediate requirement for conservations of this plant species so as to protect it from endangerment. The existing diversity is much pronounced in Wayanad and thus those populations in fragmented and inhabited area need special attention for reinforcement. *Vanda wightii* at the same time possessed more diversity in fragmented forests and its inhabited area necessitates rehabilitation/reinforcement into suitable protected forest segments so as to conserve the total diversity in safe localities.

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