



Cluster Analysis Helps to Select Appropriate Pollination States by Crossing of *Phalaenopsis* Varieties

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ARTICLE INFO

Original paper

Article history:

Received: 2 Sep 2023

Revised: 28 Oct 2023

Accepted: 8 Dec 2023

Keywords:

Breeding

Capsule's volume

Cluster

Seed weight

Sign of pollination

ABSTRACT

To study the effective morphological and physiological characteristics and the cognition of main factors affecting pollination yield and seed production of *phalaenopsis* orchid, five varieties and their crossing were studied in a greenhouse of the Agriculture Institute, Research Institute of Zabol on 2019-2022. Eight morphological and physiological characteristics of pollination were evaluated in this research for three years and their grouping was according to a completely randomized design. The results of cluster analysis with the method of UPGMA based on the maximum distance between the clusters, 25 pollination states were divided into two groups. The results of detection of function analysis significantly correlated with cluster analysis. Also, factors analysis showed that two main factors fitted 77.1% of the data variation considered in the breeding of characteristics with the most variations in each factor. Morphological characteristics such as fresh weight of capsule (FWC), capsule volume (CV), and weight of seed in capsule (WSC) had the most positive variations between factors, and physiological traits such as TCS (Time to Capsule formation Sign) and TPS (Time to first Pollination Sign) showed the most negative one. The most amounts of FWC, CV, and WSC characteristics belonged to Nottingham× Nottingham with 9.66 g, 23.5 cm³, and 3.58 g, respectively and in the cross-pollination of Andorra× Nottingham with 8.67 g, 20.8 cm³, and 3.4 g respectively. The results of the factors analysis showed the importance of morphological pollination characteristics (FWC, CV, and WSC) and physiological traits (TPS and TCS) in the indirect selection of desired genotypes for pollination of orchid varieties.

DOI: [10.22126/ATIC.2023.9725.1117](https://doi.org/10.22126/ATIC.2023.9725.1117)

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1. Introduction

The Orchidaceae is one of the largest families of flowering plants, with about 30,000 species classified into around 725 genera. The *Phalaenopsis* is a monopodial orchid in the Orchidaceae family. The genus *Phalaenopsis* comprising over 60 species belongs to the tribe Vandaeae under the subfamily Vandoideae, which contains five subgenera, viz. subgenus *Proboscidioides*, subgenus *Aphyllae*, subgenus *Parishianae*, subgenus *Polychilos*, and subgenus *Phalaenopsis* in Christenson (2001) note.

Increasing market circulation and rising trade in orchids are forcing breeders to develop varieties with unique characteristics, including flower color, morphology, and resistance using a range of approaches, including traditional and molecular

breeding (Li *et al.*, 2021). The breeding of *Phalaenopsis* orchids involves the cross-pollination of two selected parental plants. Parents with regular meiotic behavior can often produce seeds successfully. Currently, most *Phalaenopsis* hybrids are obtained after interspecific hybridization (Hsu *et al.*, 2014).

Cattell (1965) reported the factor analysis method involves reducing a large number of correlated variables to a much smaller number of clusters of variables called factors. After extraction, the factor loading matrix was submitted to a varimax orthogonal rotation, as applied (Kaiser, 1958). The amount of variance accounted for by the common factors, the array of commonality was estimated by the highest correlation coefficient in each array as suggested (Seiler and Stafford, 1985).

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Factor analysis is a powerful multivariate method for estimating yield components, extracting a subset of similar variables, understanding the basic concepts of multivariate data, recognizing biological relationships, and existing usage between characteristics, reducing a large number of characteristics that correlated to a small number of factors and explanations of the correlations between variables (Masoudi et al., 2008). Morphological characteristics as first markers can be a good way to exploit genetic diversity in breeding programs. These traits are usually low-cost and easy to evaluate without the need for advanced biochemical and molecular techniques. When characteristics have high heritability, they are considered one of the morphological characteristics and a suitable option in genetic diversity studies (Fu et al., 2008; Shao et al., 2010). Plant phenotyping is defined as the investigation of plant characters by researchers for yield, quality and resistance to biotic and abiotic stresses. Genetic variation and selection are the two basic principles of plant breeding. Hence, it is important to decompose the visible phenotypic variation into heritable and non-heritable components with suitable genetic components like the genotypic coefficient of variation, heritability and genetic advance (Begna, 2021).

Anuttato et al. (2017), in a study on morphological characteristics of *Rhynchosyilis gigantean* concluded 49 wild types of this species were divided into two groups based on cluster analysis using the UPGMA method. The first group was in the low geographic areas, and the second was in the mountainous regions. The species and hybrids were divided into two main groups in a study of 16 *phalaenopsis* species. Their hybrid species were grouped in one group and hybrids in another group based on the dendrogram (Chang et al., 2009). In a study on two species of *Passiflora* and hybrids derived from crosses by cluster analysis were divided into two separate groups, five characteristics of this flower, including internode length, stem diameter, leaf length, leaf width and leaf area that maternal rootstock, were grouped in one group and hybrids in the other. Another research was done based on morphological characteristics and evaluation of genetic variation of 50 genotypes of *Chrysanthemum*. The results of cluster analysis divided the genotypes into six groups, and the results of the detection function fitted 89.6% of the total variance of the data. Evaluation results of this study showed that some genotypes

belonging to cluster A with small plants and genotypes belonging to cluster E with large plants as a parent could initiate a new cycle of chrysanthemum breeding (Roain et al., 2015).

Statistical analysis showed that the treatment of parent material as well as the time of crossing did not show a significant effect on the time of fruit formation, except for the crossing of *Phalaenopsis* Joane Kileup June (male) x *Vanda tricolor* (female) executed at the third week after blooming which the fruit emergence appeared at the 12 days after. Moreover, this pair of crossing in its reciprocal crossing data also indicated a longer time when the crossing was executed in the third week after blooming (9 days). The fastest fruit formation was shown by the crossing pair of *Phalaenopsis* Joane Kileup June (female) and *Vanda tricolor* (male) crossed in the third week after blooming (Hartati, 2010).

Modern hybrids derive from two high-quality paternal strains. Such cultures are designed to better the size and coloring of the flowers, as well as other characteristics, like longevity, inflorescence quality, disease resistance and cultivation ease. Although the period of growth and flowering of new hybrids stretches on about six years, introducing newer hybrids on the market is essential for the orchid trade (Roxana and Maria, 2013). There are many factors affecting the success of crossing such as pollen condition (health, freshness, maturity, sterility) and compatibility level of crossing. Pollen condition is straightly important in determining the success of crossing as the first step of pollination is the unity of pollen and stigma, then the pollen will emerge to form a pollen tube to reach the ovule. The success of crossing is determined by the ratio of the emerging fruit number to the number of flowers being crossed in percentage (Hartati, 2010).

This study aimed to determine the importance of studied characteristics concerning pollination's morphological and physiological characteristics using factor analysis and grouping of pollination states to investigate genetic variation among clusters based on the traits studied to identify and determine suitable varieties and pollination states. Using these patterns leads to the design of more successful and valuable breeding programs for producing desirable hybrid varieties.

2. Materials and methods

The current research was done in a greenhouse of Agriculture Institute, Research Institute of Zabol, in 2022. Five varieties of *phalaenopsis*: Nottingham (W), Bucharest (R), Memphis (P), Dubrovnik (Y) and Andorra (K) were pollinated by an orchid pollination syringe. Plants were self- and cross-pollinated. Characteristics were measured and recorded, such as time to pollination symptoms (TPS), time to the first sign of capsule swelling (TCS), capsule length (CL), capsule volume (CV), the full weight of capsule (FWC), empty weight of capsule (EWC), the weight of seeds in each capsule (WSC) and time to capsule ripening (CR). After determining the number of groups, the analysis function was used for grouping accuracy, and intra-group similarity estimation was performed for cluster analysis based on similarity and their grouping was according to a completely randomized design. Factor analysis was used to extract the factors. The number of factors required for the descriptive characterization of the data was determined according to the eigenvalues and the percentage of total

variance justified by the various factors. Also, the scatter plot was used to determine the number of factors to minimize the number of variables that have the highest coefficient on a factor; also varimax method was used for orthogonal factor rotation to better intercept the factors. The calculations were performed using SAS 9.1 software.

3. Results

3.1. Data analysis

The maximum distance between clusters was used for cluster analysis. The varieties were grouped based on the measuring of similarity of characteristics 0-25 states. Dendrogram segmentation divided different pollination states into two groups based on the farthest average (with a dendrogram cut off 1.5-2.7). The first group consisted of Nottingham× Nottingham. The second group was divided into 5 clusters. The first cluster consisted of two pollination states (Bucharest× Bucharest and Andorra× Andorra), and the second cluster consisted of different pollination states with the closest distance to the first cluster (Fig. 1).

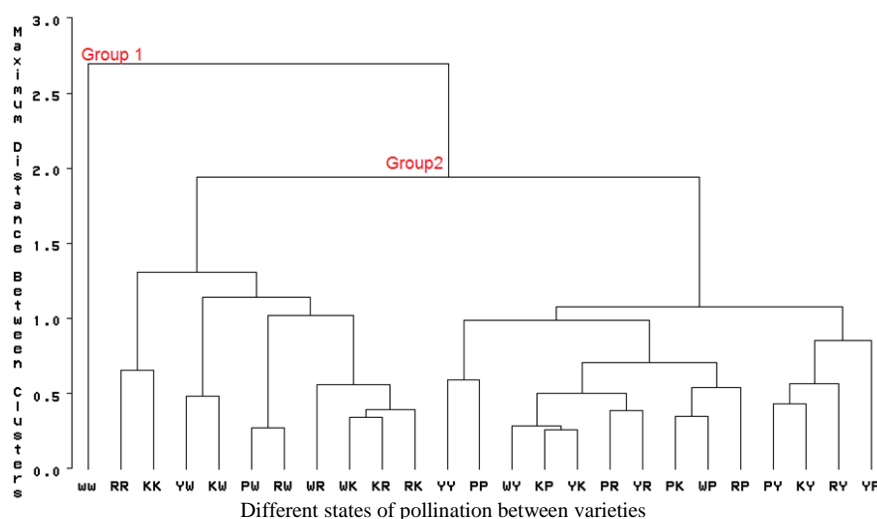


Figure 1. Grouping dendrogram based on morphological and physiological characteristics of self-pollination and cross-pollination of *phalaenopsis* orchid.

WW states (self-pollination of Nottingham × Nottingham) are separate from other pollination states based on TPS, TCS, CV, CL, FWC, and EWC characteristics. Still, there is an overlap between the first group and the second cluster of the second group in CR characteristics (Table 1).

There is an overlap between clusters 1, 2, 3, and 4 in the second groups on TPS characteristics, while there were overlaps between clusters 1 and 2; also between

clusters 3, 4, and 5 on TCS characteristics separately. There was a complete overlap in WSC characteristics between all the clusters of the second group. Also, It was observed overlap between FWC and EWC characteristics. There were overlaps between clusters 1 and 2; as well as between clusters 3, 4, and 5 separately. The results showed clusters 1, 3, 4, and 5 had overlap; and the second cluster had overlap with clusters 3, 4, and 5 (Table 2).

Table 1. Mean comparisons of characteristics based on the distribution in clusters.

Characteristic	Group 1			Group 2		
	Cluster 1	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
TPS (day)	4.6 ^e	6 ^{cd} -6.6 ^{abcd}	6 ^{cd} -7.2 ^{ab}	5.8 ^d -6 ^{cd}	6 ^{cd} -6.8 ^{abcd}	5.8 ^d -7.4 ^a
TCS (day)	13.6 ⁱ	15.8 ^{gh} -17.4 ^f	15.4 ^h -17.8 ^f	20.6 ^{cd} -22 ^b	17.4 ^f -19.8 ^{ed}	20.4 ^{ed} -24.2 ^a
CL (cm)	11.04 ^a	6.99 ^d -8.5 ^{bc}	5.62 ^{kl} -8.82 ^{bc}	5.21 ^{lm} -5.65 ^{ijkl}	5.31 ^{klm} -7.07 ^d	5.03 ^m -5.96 ^{ghij}
FWC (gr)	9.66 ^a	6.68 ^{efgh} -6.8 ^{efg}	6.46 ^{ghi} -8.67 ^b	5.53 ⁱ -6.61 ^{fgh}	6.09 ⁱ -7.11 ^e	5.22 ^j -6.38 ^{ghi}
WSC (gr)	3.58 ^a	2.55 ^g -2.77 ^{efg}	2.54 ^g -3.4 ^{ab}	2.06 ^{hi} -2.70 ^{efg}	2.05 ^{hi} -2.84 ^{efg}	1.65 ⁱ -2.05 ^{hi}
EWC (gr)	6.07 ^a	3.91 ^{jhikl} -4.25 ^{fghi}	3.88 ^{ijklm} -5.27 ^b	3.47 ^m -3.91 ^{hijkl}	3.47 ^m -4.56 ^{def}	3.53 ^{lm} -4.33 ^{efgh}
CV (cm ³)	23.5 ^a	17.6 ^{efg} -19.7 ^{bcd}	15 ^{ijkl} -20.8 ^b	12.5 ^{mno} -13.6 ^{klmn}	14.4 ^{ijklm} -17.4 ^{efgh}	11.4 ^o -13.8 ^{ijklmn}
CR (day)	121 ^{bc}	113 ^{fghi} -118 ^{cd}	107 ^{kl} -125 ^a	106 ^l -109 ^{kjl}	109 ^{kjl} -112 ^{ghij}	110 ^{ijk} -115 ^{def}

Means with the same letters had no significant differences ($P > 5\%$) from each other in the same column

The result of cluster analysis in this research conformed to other researchers' results (Santos *et al.*, 2011; Anuttato *et al.*, 2017).

Table 2. The result of the discrimination function for grouping confirmation in valuated pollination states.

Group	1	2	Sum
1	5	0	5
	100%	0	100
2	2	118	120
	1.67%	98.33%	100
Total sum	7	118	125
Estimation error for groups	5.60	94.40	100.00
Ratio	100	99.07	99.99

According to the table obtained from the discrimination function analysis, it can be concluded that the grouping correctness obtained from cluster analysis and the factor components was 99.9. Also, the results showed that the first group had 100% accuracy. Of the four multivariate tests performed to investigate the discrimination function analysis, the most amounts and F values were related to the statistical test, Roy's Greatest Root, 13.12 and 13.12 respectively that F is significant ($P \leq 0.01$). Other statistical tests such as Wilks' Lambda, Pillai's Trace, and Hotelling-Lawley Trace are significant ($P \leq 0.01$). The results of this research confirmed the result of studies on chrysanthemum flowers, that the discrimination function analysis confirmed 89.6 % of the grouping (Roein *et al.*, 2015); Of course, in this study, the grouping was confirmed by discrimination function analysis up to 99%.

3.2. Stepwise regression

The characteristic of FWC with the highest correlation with seed weight per capsule (0.89) was evaluated as the independent variable by stepwise multivariate regression (Table 3). Of the 8 studied characteristics, only two variables entered the equation.

In the first step, EWC was entered into the equation with $R^2 = 76.7\%$. Also, EWC didn't have a significant correlation with FWC. In the second step, CL entered the equation with $R^2 = 83.7\%$. However, FWC had a significant correlation with CL (0.87). Stepwise regression is used to obtain effective parameters, reduce the number of variables and increase their selection efficiency. According to the stepwise regression result, selecting the cultivar based on capsule length was more appropriate.

Table 3. Stepwise regression in 8 morphological and physiological characteristics in 25 pollination states.

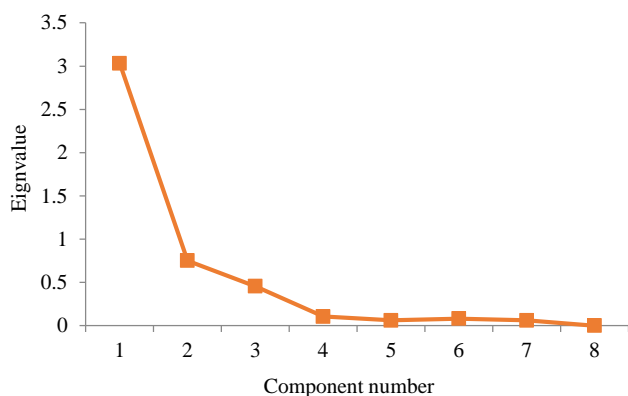
Step	Entered characteristics to the model	Intercept	X ₁	X ₂	R ²	F
1	EWC	-1.44	1.19	-	76.7	75.7**
2	CL	-3.41	1.14	0.54	83.7	9.4**

The rotational Verimax matrix coefficients in Table 3 showed that the factor analysis results confirm the cluster analysis results. Since the first two factors in factor analysis fit 77.1% of the variance in the data, we conclude that most of the data and the influence of the varieties from characteristics were summarized in these two factors. Table 4 shows Factors Analysis 1 and 2 had eigenvalues of 3.03 and 0.75, respectively. Except for Factors 3 and 4, the other factors have a coefficient factor less than 0.1. The maximum positive effect of the first factor values belonged to WSC, FWC and CV with 0.872, 0.92 and 0.87, respectively.

According to Table 4 and Fig. 2, the FWC factor has an eigenvalue affected by different varieties. After that, the WSC factor as the second factor is affected by different varieties in terms of eigenvalue with big differences. Other factors showed that they are less affected. Eigenvalue shows the importance of factors in different varieties.

Table 4. Factor analysis result for all measured characteristics.

Characteristics	FA	
	FA1	FA2
WSC	0.872	0.131
TPS	-0.432	0.807
TCS	-0.862	-0.206
CL	-0.931	0.136
FWC	0.927	0.145
EWC	0.310	-0.789
CV	0.894	0.832
CR	0.538	0.438
Eigenvalue	4.607	1.575
Proportion	57.5	19.6
Cumulative	57.5	77.1

**Figure 2. Scree plot of the relationship between factors and Eigenvalue.**

4. Discussion

Cluster analysis was performed on all characteristics measured by the UPGMA method. The best cut-off points on these two groups are the points that divide the total pollination states into two separate groups, according to the maximum distance between the merging location of separate groups from the dendrogram tree and the results of discrimination function analysis on these groups.

Analysis of variance for the two groups in total evaluated characteristics showed that the second cut-off points divided the pollination states into two groups were the most suitable cut-off points among these points. According to the analysis of variance results, the two groups were significantly different on many characteristics, especially morphological characteristics of pollination, such as CV, FWC, and WSC. Also, the result of discriminant function analysis estimated correct substitution of 99% on these two groups, as the second group, grouped 100%, consisted of clusters 1, 2, 4, and 5; and the grouping error in the third cluster was about 1%. Therefore, this grouping was selected and discussed. The first group consisted of self-pollinated Nottingham variety according to the

results of cluster analysis and comparison with the average of groups that the most average morphological characteristics included CL, WSC, CV, and FWC. The second group consisted of 5 clusters and the first cluster had two states of self-pollinated: Andorra× Andorra and Bucharest× Bucharest. The first cluster of the second group was the closest to the first group in terms of average characteristics. The second and fourth clusters of the second group had the most leaf and branching genotypes with 16 pollination states. It can be seen only cross-pollinated states in the second and fourth clusters. Self-pollinated states such as Dubrovnik× Dubrovnik and Memphis× Memphis were seen in the third cluster located more distant from the first group. The fifth cluster of the second group, which is the most distant from the first group and showed the lowest values of pollination morphological, there were cross-pollinated of Dubrovnik× Memphis and other pollination states on Dubrovnik rootstock.

Comparing the results of factor analysis showed that two main factors should be considered increased characteristics with positive correlation in varieties and decreased characteristics with negative correlation. Based on the studied characteristics, the first factor was the morphological factor, and the second factor can be considered the physiological factor of pollination. The identification or measurement of characteristic or characteristics with high yield had particular importance in breeding programs. These characteristics should be directly identified and make it easier to select and increase the efficiency of hybrids. According to the negative sign for the first factor on the characteristics such as TPS, TCS, and CL, this factor may indicate a negative association between morphological characteristics and the relationship importance of some of these characteristics with WSC. It indicates the end of the vegetative growth period due to the greater share of phonological characteristics.

The second factor was more positively affected by TPS and CV, but TCS and EWC had a negative effect on this factor. For this reason, the second factor can be called the physiological maturity factor of the plant, as can be seen between these states. Therefore, it was concluded that the gene of the mentioned characteristics is recessive in Nottingham and Andorra varieties as male parents on the Dubrovnik and Memphis as female parent rootstock. Due to the low quality and yield of pollination characteristics in the

two varieties of Dubrovnik and Memphis, their gene function was recessive, appearing high in yield by hybridizing these two varieties. Therefore, it is concluded that it can increase pollination yield (CL, FWC, EWC, and WSC) by re-modifying hybrid Dubrovnik× Memphis. Stepwise regression is also used to obtain effective parameters, reduce the number of variables, and increase their selection efficiency. According to the stepwise regression results, selecting the cultivars based on capsule length (CL) was more appropriate.

The crossing of *Phalaenopsis* sp. and Vanda tricolor was compatible, and the use of *Phalaenopsis* sp. as male parent had a better probability of producing fruits rather than the opposite (Hartati, 2010).

5. Conclusion

According to Pangestu et al. (2014), a morphological marker on plants is a process to determine the phenotypic characteristics of a plant by observing the stems, fruits, roots, leaves and flowers that cover the entire morphology of plants and knowing the genetic relationship between species. In general, varieties and hybrids were divided into two main groups by cluster analysis of effective morphological and physiological characteristics of five self-pollination and 20 cross-pollination states. In the first group, it can be seen only self-pollination of Nottingham variety by characteristics average and other pollination states are seen in the other group. The result showed that cross-pollination altered morphological and physiological characteristics, separating the average characteristics of self-pollination states from cross-pollination. Also, grouping confirmation of discriminant Analysis showed that the varieties of current research had significant genetic variation. Some genotypes could be used in breeding programs with high yield potential or other desirable characteristics and produce new and modified varieties. It can be called the morphological characteristics factor and the second factor as the physiological characteristics of pollination by the factor analysis results. The desired characteristics were CV, WSC and TPS based on the factor analysis. The selection of the superior paternal or maternal parent in orchids makes it possible to use the superior parent in polyploidy breeding programs and increase the quality of the resulting progeny. The selection of parents' quality is a very important factor in determining the

success of a hybridization program. The greater genetic diversity will increase selection effectiveness, so new varieties can be obtained with desired superior characteristics, especially can adapt to new environmental conditions outside of their natural habitat (Hartati et al., 2021).

Conflict of Interests

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval and consent to participate

No human or animals were used in the present research.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

The main part of the experiment, production stages and providing 80% of the financial cost was the responsibility of the corresponding author. Mr Karimian and Dr. Mohkami have collaborated in the data analysis.

Informed Consent

The authors declare not to use any patients in this research.

Funding/Support

This article was carried out with the support of the Agriculture Institute, Research Institute of Zabol, Zabol, Iran, and in the form of a research project (number of PR-UOZ-1401-6918-1).

Acknowledgement

This article was achieved based on the material and equipment of Agriculture Institute, Research Institute of Zabol, that the authors thanks it.

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HOW TO CITE THIS ARTICLE

Bidarnamani F., Mohkami Z., Karimian M.A. 2023. Cluster Analysis Helps to Select Appropriate Pollination States by Crossing of *Phalaenopsis* Varieties. *Agrotechniques in Industrial Crops* 3(4): 223-229. [10.22126/ATIC.2023.9725.1117](https://doi.org/10.22126/ATIC.2023.9725.1117)