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# Estimation of genetic variability of a Gerbera Brazilian collection based on morphological traits and EST-SSR markers

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**Abstract**

EST-SSRs are ubiquitous in transcribed sequences, are typically locus-specific and co-dominant, and are often multi-allelic, highly polymorphic, and transferrable among species within genera. Because morphological markers are not always available for analysis and are affected by environmental conditions, association studies using molecular and morphological data are the best choice to find informative markers useful for plant breeding. This is the first study that correlates molecular and morphological data in gerbera (Gerbera spp.) Nine quantitative and 12 qualitative morphological traits, as well as 17 EST-SSR molecular markers, were analyzed to determine the correlations between individual quantitative traits and between quantitative traits and molecular markers. They also identified new informative markers and were used to analyze the genetic variability among 32 gerbera accessions. To accomplish this goal, we added the distance matrices of morphological and molecular data. Head diameter and stem diameter had a significant and positive correlation (0.89), while head diameter and leaf length had the lowest correlation (0.53) among the morphological traits. From the 17 molecular markers, the eight that were associated with seven quantitative traits were identified. The GERB12 and GERB17 markers correlated with ray floret width and stem length, respectively. The dissimilarity dendrogram resulting from the sum of matrices showed a 3.87 average dissimilarity and 0.92 cophenetic correlation (r), allowing us to identify the most contrasting genotypes; for example wild type contrasted with many others. The present study will enable us to predict the best controlled crosses between contrasting genotypes and will allow for molecular marker-assisted selection associated with morphological traits.

**Keywords***:* Qualitative traits, quantitative traits, EST-SSR, Gerbera jamesonii hybrid.
**Abbreviations:** EST-SSR_expressed sequence tag–simple sequence repeat; LOD_logarithm of the odds; RAPD_random amplified polymorphic DNA; UPGMA_Unweighted Pair Group Method with Arithmetic Mean.

## Introduction

The African daisy (*Gerbera hybrida*) is an ornamental plant native to South Africa, Madagascar, and tropical Asia (Bremer 1994). Gerbera cultivars are grown commercially worldwide. Their flowers are durable and attractive, presenting a wide variety of colors, which is one of the main morphological traits of agronomic interest, including for breeding programs (Rezende, 2005). The modern cultivars of hybrid gerbera mostly result from artificial crossings between *G. jamesonii* and *G. viridifolia*. Spontaneous hybrids between these two species have not yet been found (Kloss et al. 2005; Sane and Gowda 2005). The analysis of genetic variability in gerbera is a prerequisite for breeding programs because it can generate data on the genetic relationships existing among the genotypes of this genus. Among the strategies available for evaluating genetic variability, the use of molecular markers is the most widely applicable because these markers are best suited for understanding the genome and may be used in paternity testing, genetic variability characterization, the elucidation of genetic relationships between genotypes, developing methods for the maintenance of genetic variability existing in germplasm banks, and identifying genes or combinations of features related to key traits of biological and agronomic interest (Hayden et al. 2010). Another approach to studying genetic variability is the analysis of morphological and phenotypic traits because these methods are relatively simple to perform. The analysis of variability based on this type of feature alone is not conclusive given the limited number of traits, the strong effect of environmental factors, and the plant development stage. Morphophenological characterization does not replace molecular analyses but may be used to supplement both characterization and genetic variability studies and the development of cultivars (Fufa et al. 2005). In contrast, molecular markers based on DNA sequence polymorphisms are little affected by environmental factors and show high polymorphism rates. While morphological markers reflect the variation in the coding regions of the genome, DNA-based molecular markers represent variations that occur in various regions of the genome, including coding and non-coding regions. Thus, molecular markers provide a fast and reliable method to estimate genetic relationships between genotypes (Thormann et al. 1994; Tatikonda et al. 2009). Molecular

characterization has been widely used to quantify the genetic variability existing among different accessions comprising the germplasm banks (Glaszmann et al. 2010), enabling researchers to clarify the genetic structure and diversity in a wide range of plant species (Leišová et al. 2007; Kilian et al. 2007). EST-SSR markers were developed from expressed sequence tags (ESTs) generated by multiple transcriptome sequencing projects. In addition to the codominant feature, they have more advantages than other types of molecular markers because they are part of a translated region and can control the expression of a trait or simply be associated with a trait of interest (Thiel et al. 2003). These types of sequences are more highly conserved than non-coding sequences of the genome, like SSR sequences. Thus, these EST-derived markers have great potential to be transferred to related species (Thiel et al. 2003; Varshney et al. 2005). Compared to morphophenological trait analysis, EST-SSR molecular markers are more informative, but when the two types of markers are used together, the analysis becomes even more powerful. This approach has been used in several species, including Fragaria x ananassa Duch (Conti et al. 2002), *Allium sativum* L. (Mota et al. 2006), *Prunus* sp. (Kadkhodaei et al. 2011), and *Olea europaea* L. (Zaher et al. 2011). Considering that EST-SSRs are located inside translated regions, there are more possibilities of these markers being associated with a trait of interest. EST sequences, molecular markers, and genetic linkage maps have been developed and used in quantitative trait loci studies, mapping studies, genetic diversity analyses, and comparative genomics in the genus Capsicum (Yi et al. 2006; Miura et al. 2012). The only disadvantage in using microsatellites is that prior data on the sequence is necessary for the development of primer combinations (Khar et al. 2011). No studies to date on genetic diversity in gerbera have been conducted that use both molecular and morphological markers. The utility of using both molecular and morphological markers has been demonstrated in other species including cotton (*Gossypium hirsutum* L.) (Tatineni et al. 1996), common bean (*Phaseolus vulgaris* L.) (Duran et al. 2005), peanut (*Arachis hypogea* L.) (Ferguson et al. 2004), and *Cucurbita pepo* L. Ferriol et al. 2003). Multivariate analysis methods have been effectively used in the analysis, description, and selection of multiple morphological and molecular traits simultaneously, resulting in savings of time and financial resources. These benefits occur because several variables are often redundant because they are correlated, or the variables are expendable because they represent a small fraction of the total variation (Alves et al. 2003; Cruz et al. 2004; Oliveira et al, 2009). In that context, the present study aimed to quantify the correlations between individual quantitative morphological traits and between morphological traits and molecular markers, as well as to identify preferred traits to select when breeding new genotypes of *Gerbera* spp.

**Results and Discussion**

***Correlations between quantitative traits***

One of the main goals of genetic breeding programs of plants is to select superior genotypes. Thus, the evaluation of traits of agronomic interest and the estimation of the correlations existing between them is useful because it enables researchers to identify which traits affect which other trait(s) and which ones may contribute most significantly in the selection of superior genotypes for a given trait. Significant correlations were found in this study, at a 1% probability using a t-test, between all of the quantitative descriptors analyzed (Table 1). The correlation between head diameter and stem diameter was the highest (0.89). Given this result, the selection of plant materials with a larger stem diameter is expected to yield genotypes that produce larger head sizes. Both traits are of great importance for the flower market: the stem diameter is important because it increases the resistance of flowers to tipping over due to wind, and the head size is important because it determines the value of cut flowers in the ornamental plants sector. Amorim et al. (2008) noted a 0.48 correlation between the head and stem diameters in a study of the genetic correlations between 13 sunflower genotypes. The same authors found a significant correlation between stem length and leaf number (0.73) and between head diameter and seed weight (0.59). Sowmya et al. (2010) also found a positive correlation between sunflower head size and seed weight. According to Castro and Farias (2005), well-developed heads tend to have a higher proportion of larger and heavier achenes. According to Alkio et al. (2003), heavier achenes have more time to fill, enabling greater nutrient intake. The selection of a trait may knowingly or unknowingly result in a selection for or against a second trait. In some cases, the correlations may be useful; for example, De Jong and Garretsen (1985) estimated a positive and significant genetic correlation between the number of flower stems in gerbera (which requires a longer growth period for evaluation) and the number of lateral shoots (which can be evaluated in the early stage). This type of correlation is classified as indirect; that is, it is based on the evaluation of a simple trait or a trait that has the highest estimate of heritability in the broad sense, enabling us to relate it to a complex trait. Thus, the early expression of plant traits that are correlated with late-expression traits is useful for selection in genetic breeding programs. Studies on the nature and magnitude of existing correlations between morphological traits are convenient because breeding aims to improve the genetic material, not for single traits but for a set of traits simultaneously (Vencovsky and Barriga 1992). According to Cruz (1997), a favorable genetic correlation between two traits enables gains in one of them using indirect selection of the other. Nonetheless, caution is advised to avoid undesirable changes in other traits when selecting for one trait that is negatively correlated with some traits and positively correlated with others; that is, breeding for a given trait can negatively affect another trait of interest. The advent of molecular markers and the improvement of genetic and statistical methods for the analysis of molecular data enabled researchers to improve their understanding of the inheritance of quantitative traits. The identification of quantitative trait loci using molecular markers has several applications, such as increasing the efficiency of genetic breeding programs, especially for low-heritability traits (Lander and Botstein 1989), understanding of the evolution of these traits, and identifying new genes of interest (Falconer and Mackay 1996).

***Associations between EST-SSR markers and quantitative traits***

The usefulness of EST-SSR markers arises from their close linkage to potentially important genes, making them useful in identifying candidate genes for quantitative trait loci (QTL). Moreover, these markers could also be of great assistance for comparative studies in related species due to high heterologous conservation (Talia et al. 2010). Were identified in this study eight molecular markers [logarithm of the odds (LOD) ≥ 3] associated with seven quantitative traits (Table

**Table 1.** Estimates of Pearson's correlation coefficients between the quantitative traits evaluated in 32 gerbera accessions.

| Trait | LL | DFW | RFL | RFW | LN | SL | SD | HD | HN |
|---|---|---|---|---|---|---|---|---|---|
| LL | - | 0.85** | 0.80** | 0.77 | 0.73 | 0.68 | 0.60 | 0.53 | 0.61 |
| DFW | | - | 0.80** | 0.79 | 0.73 | 0.70 | 0.72 | 0.58 | 0.65 |
| RFL | | | - | 0.80** | 0.79 | 0.77 | 0.69 | 0.72 | 0.70 |
| RFW | | | | - | 0.84** | 0.88** | 0.83** | 0.80** | 0.86** |
| LN | | | | | - | 0.88** | 0.78 | 0.80** | 0.82** |
| SL | | | | | | - | 0.81** | 0.83** | 0.84** |
| SD | | | | | | | - | 0.89** | 0.88** |
| HD | | | | | | | | - | 0.85** |
| HN | | | | | | | | | - |

** Significantly different at 1% probability according to a t test. LL: leaf length; DFW: disc floret width; RFL: ray floret length; RFW: ray floret width; LN: leaf number; SL: stem length; SD: stem diameter; HD: head diameter; HN: head number.

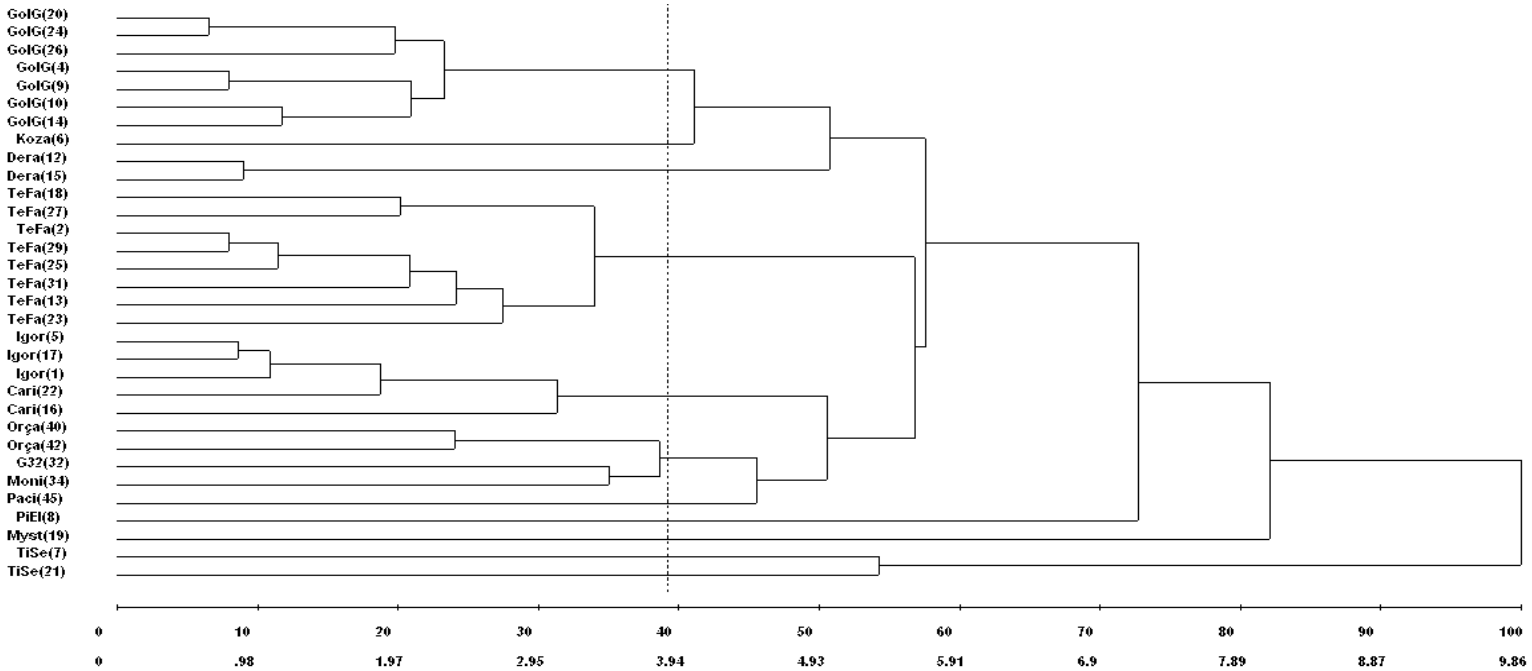


**Fig 1.** Clustering dendrogram of 32 gerbera accessions, generated by the UPGMA method, based on the analysis of morphological traits (quantitative and qualitative) and EST-SSR markers. MD: mean dissimilarity.

**Table 2.** Associations between EST-SSR markers and quantitative traits in *Gerbera* spp.

| Marker | Quantitative trait | LOD |
|---|---|---|
| GERB1 | Stem length | 3.18 |
| GERB4 | Leaf number | 3.78 |
| | Head number | 5.31 |
| GERB9 | Stem diameter | 4.38 |
| GERB10 | Leaf number | 3.39 |
| | Stem length | 5.24 |
| | Stem diameter | 6.36 |
| | Head diameter | 3.15 |
| GERB12 | Ray floret length | 5.10 |
| | Ray floret width | 8.73 |
| | Leaf number | 6.72 |
| | Stem diameter | 3.43 |
| GERB17 | Ray floret length | 5.54 |
| | Ray floret width | 7.69 |
| | Leaf number | 7.01 |
| | Stem diameter | 3.48 |
| | Stem length | 8.45 |
| | Stem diameter | 7.68 |
| GERB22 | Stem diameter | 3.39 |
| GERB42 | Head diameter | 3.15 |

**Table 3.** Accessions of gerbera evaluated in this study and their inflorescence colors.

| Code | Accession | Inflorescence color | Code | Accession | Inflorescence color |
|---|---|---|---|---|---|
| 1 | Igor | Pink, light center | 19 | Mystique | Orange, black center |
| 2 | Terra Fame | Yellow, light center | 20 | Golden G. | Yellow, black center |
| 4 | Golden G. | Yellow, black center | 21 | Wild Type | Orange |
| 5 | Igor | Pink, light center | 22 | Cariba | Red, light center |
| 6 | Kozak | Dark orange, black center | 23 | Terra Fame | Yellow, light center |
| 7 | Wild Type | Red | 24 | Golden G. | Yellow, black center |
| 8 | Pink Elegance | Pink, light center | 25 | Terra Fame | Yellow, light center |
| 9 | Golden G. | Yellow, black center | 26 | Golden G. | Yellow, black center |
| 10 | Golden G. | Yellow, black center | 27 | Terra Fame | Yellow, light center |
| 12 | Deranagem | Pink, black center | 29 | Terra Fame | Yellow, light center |
| 13 | Terra Fame | Yellow, light center | 31 | Terra Fame | Yellow, light center |
| 14 | Golden G. | Yellow, black center | 32 | G32 | Yellow |
| 15 | Deranagem | Pink, black center | 34 | Monique | Red, light center |
| 16 | Cariba | Red, light center | 40 | Orça | Completely white |
| 17 | Igor | Pink, light center | 42 | Orça | Completely white |
| 18 | Terra Fame | Yellow, light center | 45 | Pacific | Completely white |

2). The markers with the highest LOD scores were GERB12 (8.73) and GERB17 (8.45), which were related to the traits ray floret width and stem length, respectively. The GERB10 and GERB42 markers, linked to the head diameter trait, had the lowest LOD scores (3.15 for each). The use of molecular markers in modern breeding programs is based on the principle that if one gene, or a set of genes, is closely associated with an easily-identifiable molecular marker, then the selection for that marker will be more efficient for the trait itself (Hayward et al. 1994). The combined analysis of molecular and morphological markers has been performed in many ornamental species (Abe et al. 2002; Dugo et al. 2005; Dunemann et al. 1999; Yagi et al. 2006; Yan et al. 2007; Remay et al. 2009; Oyant et al. 2008; Zhang et al. 2011) but not yet in gerbera. The breeder of a genetic breeding program must have extensive knowledge of the genetic variability available within the associated germplasm bank. Traditionally, cultivars have been identified and characterized using morphological descriptors; however, developing hybrid combinations based only on the evaluation of traits of agronomic importance may complicate the choice of parent plants with which to prepare genetically viable segregating populations.

***Genetic diversity based on the analysis of morphological traits (quantitative and qualitative) and EST-SSR markers***

The technology of molecular markers offers several advantages for the identification of new cultivars in breeding programs. Here, we evaluated morphological traits (quantitative and qualitative) and molecular traits based on EST-SSR markers developed by Benemann et al. (2012),

**Table 4.** Quantitative and qualitative traits evaluated in gerbera accessions.

| Nº | Character | Mensuration unit | | |
|---|---|---|---|---|
| | **Quantitative data** | | | |
| 1 | Leaf length | cm | | |
| 2 | Disc floret width | mm | | |
| 3 | Ray floret length | mm | | |
| 4 | Ray floret width | mm | | |
| 5 | Leaf number | unit | | |
| 6 | Stem length | cm | | |
| 7 | Stem diameter | mm | | |
| 8 | Head diameter | cm | | |
| 9 | Number of heads | unit | | |
| | **Qualitative data** | | | |
| 1 | Depth of the insertions of the middle portion of the leaf | shallow (3) | medium (5) | deep (7) |
| 2 | Pubescence in the upper side of the leaf | present (1) | absent (2) | |
| 3 | Intensity of the green color on the upper side of the leaf | light (1) | dark (2) | |
| 4 | Level of the ray floret apex relative to the perianth apex | below (1) | at the same level (2) | above (3) |
| 5 | Shape of the ray floret | narrow elliptical (1) | narrow oval (2) | |
| 6 | Shape of the ray floret apex | sharp (1) | rounded (2) | |
| 7 | Number of colors of the ray floret | one (1) | two (2) | |
| 8 | Dark disc | absent (1) | present (2) | |
| 9 | Main color of stigma (CS) | white (1) | yellow (2) | orange (3) |
| | | pink (4) | red (5) | red (5) |
| | | purple (6) | brown (7) | |
| 10 | Main color of anthers | yellow (1) | orange (2) | pink (3) |
| | | red (4) | purple (5) | brown (6) |
| 11 | Floret color | yellow (1) | white (2) | red (3) |
| | | orange (4) | pink (5) | |
| 12 | Head type | simple (1) | semi-folded (2) | folded (3) |

aiming to add up the distance and genetic diversity matrices generated by the analysis of morphological and molecular data, respectively, to obtain more accurate results on the genetic relationships between the gerbera accessions characterized in this study. Genetic similarity matrices of qualitative and quantitative traits and molecular data are shown in suppl. Tables 1, 2, and 3. The dendrogram resulting from the sum of genetic matrices showed a 3.87 mean dissimilarity and 0.92 cophenetic correlation (r). Fig. 1 shows the formation of 11 groups. The first group consisted of all Golden G. accessions, which have semi-folded and yellow inflorescences. The second group consisted of only the Kozak accession (6), which has semi-folded and orange inflorescences. A 1.0 value of genetic similarity between the accessions Kozak and Golden has been found in a study of genetic variability conducted using only EST-SSR molecular markers (Benemann et al. 2012), compared to 0.98 in the present study. The third group consisted of Deranagem accessions, which have semi-folded and pink inflorescences. All Terra Fame accessions were in the fourth group, which have simple and yellow inflorescences. The fifth group included the Igor and Cariba accessions, both with semi-folded inflorescences, although Igor is pink and Cariba is red. The sixth group consisted of Orça, G32, and Monique, which have white, yellow, and red colors, respectively and simple (Orça and G32) and semi-folded inflorescences (Monique). The other accessions, Pacific, Pink Elegance, Mystique, and Wild Type, were individually grouped. The Mystique and Wild Type accessions (21) have simple and orange inflorescences, whereas Pink Elegance is semi-folded and pink, and the Wild Type accession (7) is simple and red. The Wild Type accessions (groups 10 and 11), which are not

commercially available, were the most distant from the other accessions. According to Belaj et al. (2011), ongoing research on the use of wild progenitors in a breeding program suggests high levels of heterosis in their progeny, so this could be a promising parental line for use in gerbera breeding. The similar accessions are clones of one cultivar, albeit with a certain genetic distance from each other, representing specimens of a lineage derived from vegetative propagation. The fact that they are not identical may be related to mutations after several vegetative propagation cycles. The genetic variability of gerbera detected in our study was similar to that detected by Cardoso et al. (2009) and Benemann et al. (2013), who used qualitative and quantitative descriptors, including some also used in this study. Da Mata et al. (2009) used RAPD markers for the genetic variability analysis, whereas Benemann et al. (2012) and Gong and Deng (2012) analyzed the genetic variability using EST-SSR markers developed for gerbera. The analysis of morphological traits is a common step in plant breeding for parental selection and the first choice for describing and classifying germplasm. Morphological trait analysis is also inexpensive. However, molecular analysis remains an essential tool used to examine the variability within and between genotypes and to detect geographical and environmental effects on morphological traits. Furthermore, molecular data are useful for validating morphological models. Therefore, molecular and morphological analyses are complementary tools in the characterization of gerbera accessions and are valid for identifying new cultivars. According to D'Imperio et al. (2011), the two methods are incomplete because the molecular data are not as important for the identification of cultivars by farmers and agronomists compared to phenotypic data. In contrast, morphological data are incomplete without a determination of the molecular basis of a trait, given the variations in environmental pressures.

## Materials and Methods

### *Plant materials*

The plant material evaluated in present study came from a germplasm bank at the company Pro Vitro LTDA-SP (São Paulo, Brazil) (Table 3).

### *Morphological characterization*

The accessions were evaluated based on the characterization of 21 morphological traits, including 9 quantitative and 12 qualitative traits (Table 4).

### *Molecular characterization*

Total genomic DNA was extracted using the method described by Doyle and Doyle (1987). Seventeen EST-SSR markers (Suppl. Table 4) developed by Benemann et al. (2012) were used in this study. The procedures used in DNA amplification by polymerase chain reaction (PCR) and the separation of amplified fragments by polyacrylamide gel electrophoresis were as described by Benemann et al. (2012).

### *Data analysis*

### *Molecular and morphological traits correlation analysis*

Correlations between molecular markers and morphological traits were quantified by mark simple maximum likelihood analysis (Liu 1998) using GQMol software (Cruz and Regazzi 2007), with $p < 0.05$ corresponding to a LOD score > 3.0. (LOD scores are associated with the distribution of chi-square values.) Furthermore, the estimated correlation coefficients between quantitative traits were calculated using Pearson's method, which was described by Steel and Torrie (1960). The Genes software (Cruz 1998) was used in the correlation analyses, applying the t-test at a 1% probability of error to test the significance of the correlation coefficients.

### *Multivariate data analysis*

The quantitative traits were converted to qualitative data. The conversion was based on the standard deviation of the mean. In other words, two categories were used: positive (≥ mean) and negative values from the mean (< mean), generating a binary matrix. Subsequently, the quantitative data were submitted to multivariate data analysis. The coefficient used to obtain the similarity matrix was Simple Matching (Sokal and Michener 1958). After the cluster analysis was conducted with the method UPGMA, the multivariate data analysis was conducted to join the genetic similarity matrix of morphological traits (qualitative and quantitative) and the molecular data. The results were presented in a dendrogram. NTSYS-Pc Ver. 2.1 (Rohlf 2001) software was used to perform the analysis described above.

## Conclusion

This study is the first to report the combined analysis of morphological and molecular traits related to genetic dissimilarity in the *Gerbera* genus. Our data have an immediate potential application in the selection of new genotypes and will enable advanced molecular studies on the identification of new molecular markers and their combination with morphological traits that are widely applicable to gerbera breeding. The results of this study should be valuable to gerbera genetic breeding programs because they can help guide breeders in conducting controlled crosses and selecting promising progenies.

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