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REGULAR PAPER

Morphometric analysis of *Milla biflora* (Asparagaceae: Brodieaoideae), with an identification key for *Milla*

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Background and aims – The taxonomical delimitation of *Milla biflora* has been controversial and is possibly associated with the high morphological variation of this taxon. The aims of this study were to find morphological characters that allow to distinguish groups among *M. biflora* and to analyse the morphological variation, with the purpose of generating an identification key.
Methods – A multivariate analysis was based 24 quantitative characters of nine species of the genus *Milla*. Nine morphometric characters that had the highest loadings in the multivariate analysis plus 18 qualitative characters were used to run a cluster analysis.
Key results – Discriminant analysis showed that the quantitative characters selected by the canonical discriminant analysis do not allow distinguishing all the species studied. Only the combination of qualitative and quantitative characters favours the separation of the species through the cluster analysis. Cluster analysis allowed the recognition of 35 groups with a dissimilarity value > 0.80. Twenty-six morphotypes differ from the other *Milla* species, previously recognized, in at least one character. An identification key for *Milla*, based on the diagnostic characters retrieved, distinguishes eight *Milla* species, as well as 26 morphotypes of *M. biflora* s. str. *Milla biflora* s. str. can be identified by its fistulose leaves, a pedicel (1.5–3 cm long) lacking an evident articulation, white flowers that are open during the day and night, three tepal nerves, elliptic outer tepals, narrowly elliptic inner tepals with widely attenuated base, filaments less than 1 mm long and yellow anthers. It is geographically located south to the Mexican Plateau and in part of the Trans-Mexican Volcanic Belt, where the type locality is situated.

Key words – Multivariate analysis, Asparagales, *Milla* complex, Taxonomy, Trans-Mexican Volcanic Belt.

INTRODUCTION

Cavanilles (1793) described *Milla biflora* Cav. using corms cultivated in the Real Jardín Botánico (Madrid), sent from Mexico by Sessé and Mociño. These corms belong to part of the collections performed during the Real Expedición Botánica to Nova Spain (1787–1803), in the vicinity of the actual Mexico City (McVaugh 1977). *Milla biflora* is the type species of *Milla* and following its description it has underwent several non-validated taxonomical changes (Willdenow 1799: 62, Knowles & Westcott 1839, Rafinesque 1836, Nees von Esenbeck & Schauer 1847: 703). The genus' representatives are herbaceous geophytic plants with corms and fragrant white flowers. They grow in sites with elevations between 10 and 2,800 m a.s.l., in tropical dry and temperate forests. *Milla* is near-endemic to Mexico, with *Milla biflora* also extending into Guatemala, Honduras, Arizona, Texas and New Mexico. The other ten species of *Milla* have a more restricted distribution in Mexico mainly in the Mexican Plateau, the Rio Balsas Basin and Sierra Madre del Sur. The genus has not been found in the Yucatan peninsula and in the northeast (Tamaulipas state) of Mexico. The genus is currently placed in the Asparagaceae (Angiosperm Phylogeny Group 2016).

In his review of the *Milla* complex, Moore (1953) asserted that *M. biflora* has three tepal nerves, subsessile anthers and filaments not larger than the anthers. The same author indicated that populations in southern Mexico and Guatemala show variation in diagnostic characters, which he did

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not consider to be sufficient for segregating those southern populations. Other authors examined the morphological diversity of *M. biflora* and segregated new species from materials previously determined as *M. biflora* (Ravenna 1971, Howard 1999, Gutiérrez & Solano 2015). In a recent molecular phylogenetic analysis (Gándara et al. 2014), *M. biflora* was shown to be polyphyletic. An evaluation of the morphological variation of *M. biflora* might therefore produce new taxonomic insights.

Multivariate statistics and cluster analyses for quantitative as well as qualitative characters has allowed to clarify the delimitation of several angiosperm species or species complexes (Birch et al. 1985, Chung & Kang 1994, Thompson & Lammers 1997, van den Berg et al. 1998, Levin 1998, Henderson & Ferreira 2002, Henderson 2004, Ferreira Da Costa et al. 2009, Hernández-Hernández et al. 2009, Arroyo-Cosultchi et al. 2010, Martínez-Cabrera et al. 2011, Hejazi et al. 2012, Jakovljević et al. 2013, Sánchez et al. 2013). In this study, we test the hypothesis that *M. biflora* is polyphyletic and hence should be split into several species with the aid of multivariate and cluster analyses. In addition, we use the results of our analyses to generate an identification key at species level for *Milla*. A molecular phylogenetic investigation was conducted in parallel with the present study (Gutiérrez et al. submitted).

MATERIALS AND METHOD

A total of 501 herbarium (7.4%) and field-collected (92.6%; electronic appendix) specimens belonging to nine of the eleven species of *Milla* were investigated. *Milla delicata* H.E.Moore and *M. mortoniana* H.E.Moore were not included. This study used the name of *M. biflora* for referring to all populations recognized as such by previous authors (Moore 1953, Galván 2001).

Evaluated characters

Twenty-four characters were measured (table 1) using a steel ruler and a Nikon SMZ-2T stereoscopic microscope with an adapted Vernier. Eighteen qualitative characters of the leaf, inflorescence axis and flower and their corresponding states were recognized. Nine of them were binary (table 2). The character states for leaf shape in cross-section, length of epidermal prominences and other lamina anatomical characters follow Gutiérrez et al. (2015). Since these epidermal prominences seen under the stereoscopic microscope correspond to denticulations; here we used this term plus the size.

Multivariate analyses

It was proved that the 24 quantitative characters fulfilled the assumptions of multivariate statistics, such as normality and homogeneity of variances with variables log (base 10) transformed. A principal component analysis (PCA) was performed in order to detect the variables that account for the greater percentage of variation. Thirty-four groups were established based on qualitative characters previously evaluated in populations observed in the field and in herbarium specimens, with the objective of testing the morphological delimitation of *M. biflora* (table 3). A canonical discriminant analysis (CDA) was performed in order to identify the subgroup of characters that support the separation of the nine species and 34 groups *a priori* established. Additionally, whether the groups were statistically different was evaluated through Mahalanobis distance. A canonical discriminant analysis (DA) allowed verifying how many individuals can be correctly classified under any given species or group. All analyses were performed on the statistical package SAS (SAS Institute 2008).

Table 1 – Morphological characters used for the morphometric analyses of *Milla*.

No.	Character
1	Corm length
2	Corm width
3	Corm scales length
4	Leaf length
5	Leaf width
6	Inflorescence basal axis length
7	Inflorescence basal axis width
8	Pedicel length
9	Bract length
10	Bract width
11	Floral tube width
12	Floral tube length
13	Outer tepal width
14	Outer tepal length
15	Inner tepal width
16	Inner tepal length
17	Apiculum length
18	Filament length
19	Anther length
20	Anther width
21	Ovary length
22	Ovary width
23	Style length
24	Gynophore length

Cluster analysis

A mixed data matrix was generated, with eighteen qualitative characters (table 2) and mean values of the nine quantitative characters selected in the CDA analysis because of their larger weights for separating groups. The matrix was standardized in standard deviation units to maintain the principle of equal weighting between characters (Sneath & Sokal 1973). Through this matrix, a similarity matrix was generated by means of the Euclidean distance coefficient, and the method of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) served as a clustering algorithm. These analyses were performed with the NTSYS software version 2.1 (Rohlf 2000).

Table 2 – Qualitative characters used in the cluster analysis of the *Milla* species and of *Milla biflora* s. lat.

	Character	Character state
1	Corm with rhizomes	absent = 0, present = 1
2	Leaf shape in cross section	widely elliptic = 0, circular = 1, widely depress = 2, transversally elliptic = 3, obovate depress = 4, narrowly transversally elliptic = 5, flat = 6
3	Adaxial leaf surface	flat = 0, round = 1
4	Leaf striations	absent = 0, present = 1
5	Leaf denticulations	absent = 0, present = 1
6	Leaf denticulations length	short (< 50 µm) = 0, long (≥ 50 µm) = 1
7	Number of vascular bundles	six = 0, seven–twelve = 1, more than twelve = 2
8	Leaf mesophyll	compact = 0, collapsed = 1
9	Fibers wall associated to the vascular bundle	thick = 0, thin = 1
10	Indument in the inflorescent axis	scabrous = 0, pilose = 1, smooth = 2
11	Floral articulation	present = 0, absent = 1
12	Number of flowers	one = 0, two = 1, three or more = 2
13	Flower opening	nocturnal = 0, diurnal-nocturnal = 1
14	Outer tepal shape	narrowly elliptic = 0, elliptic =1, widely ovate = 2
15	Inner tepal shape	narrowly elliptic = 0, elliptic = 1, ovate = 2
16	Inner tepal base	attenuated = 0, abruptly attenuated = 1, widely attenuated = 2
17	Number of nerves in the tepals	three = 0, five = 1, seven–twelve = 2
18	Anther colour	yellow = 0, blue =1, green = 2

Table 3 – Species and morphotypes of *Milla*, and states of the Mexican Republic or geographical region where they are distributed. EDM = Estado de México; SLP = San Luis Potosí, BCS = Baja California Sur. States with more than one morphotype are identified with an Arabic number. The letter in parenthesis identifies the species and morphotypes in figure 1 (*species not included).

Species/Morphotype	Distribution	Morphotype	Distribution
M. biflora (i)	Aguascalientes	Morphotype 10 (R)	Michoacán1
M. biflora (i)	Guanajuato	Morphotype 11 (S)	Chiapas1
M. biflora (i)	Morelos2	Morphotype 12 (T)	Chiapas2
M. biflora (i)	Puebla; Veracruz	Morphotype 12 (T)	Oaxaca8
M. biflora (A)	Mexican Basin	Morphotype 13 (U)	Oaxaca11
M. bryanii (B)	Coahuila	Morphotype 14 (V)	Oaxaca12
M. filifolia (C)	EDM; Morelos	Morphotype 15 (W)	Oaxaca7
*M. magnifica**	EDM; Guerrero	Morphotype 16 (X)	Oaxaca10
M. mexicana (D)	Morelos; Puebla	Morphotype 17 (Y)	Oaxaca13
M. oaxacana (E)	Oaxaca	Morphotype 18 (Z)	Puebla1
M. potosina (F)	Guanajuato; SLP	Morphotype 19 (a)	Guerrero
M. rosea (G)	Nuevo León	Morphotype 20 (b)	Oaxaca; Puebla2
M. valliflora (H)	Oaxaca; Puebla	Morphotype 21 (c)	Oaxaca1
Morphotype 1 (I)	Oaxaca2	Morphotype 22 (d)	Oaxaca3
Morphotype 2 (J)	Morelos1	Morphotype 22 (d)	Oaxaca4
Morphotype 3 (K)	BCS	Morphotype 23 (e)	Zacatecas1
Morphotype 4 (L)	Oaxaca5	Morphotype 24 (f)	Nayarit2
Morphotype 5 (M)	Zacatecas3	Morphotype 25 (g)	Chihuahua
Morphotype 6 (N)	Nayarit1	Morphotype 25 (g)	Michoacán2
Morphotype 7 (O)	Puebla2	Morphotype 25 (g)	Zacatecas2
Morphotype 8 (P)	Oaxaca6	Morphotype 26 (h)	Hidalgo
Morphotype 9 (Q)	Oaxaca9		

Table 4 – Results of Principal component analysis (PCA) on 24 quantitative characters of species and morphotypes of *Milla*.
[a] Characters with the highest contribution.

Component	1	2	3	4	5
Eigenvalue	7.10	3.15	1.69	1.46	1.22
Percentage variation (%)	29.59	42.72	49.77	55.87	60.96
Corm length	0.081	0.205	0.047	0.047	-0.258
Corm width	0.243[a]	-0.202	0.167	0.078	-0.061
Corm scales length	0.188	-0.069	0.331	0.253	-0.111
Leaf length	0.111	0.064	0.323	0.434[a]	0.090
Leaf width	0.245[a]	-0.260	-0.009	0.136	-0.031
Inflorescence basal axis length	0.244[a]	-0.340[a]	0.054	-0.059	0.076
Inflorescence basal axis width	0.268[a]	-0.166	0.067	0.209	0.075
Pedicel length	0.271[a]	-0.293	0.129	-0.050	0.074
Bract length	0.159	-0.014	-0.176	0.131	0.624[a]
Bract width	0.206	0.217	-0.002	0.198	-0.270
Floral tube width	0.193	0.056	0.113	0.100	-0.192
Floral tube length	0.162	0.107	0.358[a]	-0.407	0.081
Outer tepal width	0.183	0.162	-0.033	0.034	-0.384
Outer tepal length	0.241	0.120	-0.173	-0.254	0.081
Inner tepal width	0.283[a]	0.198	-0.210	0.040	0.048
Inner tepal length	0.188	0.187	-0.068	-0.252	-0.057
Apiculum length	0.280[a]	0.188	-0.202	0.036	0.061
Filament length	0.086	0.254	-0.276	0.293	0.295
Anther length	0.184	-0.263[a]	-0.263	-0.090	-0.096
Anther width	0.174	0.258	-0.093	0.051	-0.112
Ovary length	0.071	0.338[a]	0.288	0.028	0.211
Ovary width	0.240	0.062	0.134	-0.199	0.101
Style length	0.167	0.099	0.201	-0.404[a]	0.127
Gynophore length	0.207	-0.093	-0.380[a]	-0.086	-0.207

RESULTS

Character variation

The PCA revealed that fourteen characters concentrate the greatest variation in the first five components with 60.69% of the accumulated variation (table 4). These fourteen characters showing the highest values in the PCA were used in the CDA to test the 34 groups of *M. biflora* previously established based on qualitative characters (table 3). The CDA allowed the identification of the variables providing the greater information to discriminate among the *Milla* species and from the 34 groups of *M. biflora*. More than 91% of the total variation was accounted by five canonical functions (table 5). The graphic representation of the three canonical functions revealed a greater separation of the species and morphotypes (fig. 1) when eliminating *M. magnifica* H.E.Moore, the species with the largest size in the evaluated characters. The DA showed that 80% of the individuals from four species of *Milla* plus seven morphotypes can be recovered with quantitative characters, and for the remainder species and morphotypes, the percentage was much lower (table 6).

Cluster analysis

This analysis allowed the separation of the *Milla* species and most of the morphotypes. *Milla rosea* H.E.Moore from Nuevo León was recovered as the most dissimilar species based on floral tube < 5 cm long and inner tepal < 5.8 mm wide. Additionally, two groups were recovered (fig. 2). Group A includes only three morphotypes showing similarity in the floral tube of 5–8.5 cm long and an abruptly attenuated inner tepal base (fig. 3A). Group B groups the species and morphotypes distinguished by having an attenuated or widely attenuated inner tepal base (fig. 3B). *Milla bryanii* I.M.Jonst. is the species which shows the smallest Euclidean distance (0.80). Based on this value, morphotypes with values below it (0.80), where considered as a single entity, as is the case for morphotype 12 [Chis2, Oax8], morphotype 22 [Oax3, Oax4], morphotype 25 [Chih, Mich2, Zac2] and *Milla biflora* Cav. s. str. [Ags, Gto, Ver-Pue, Mor2, VM] (fig. 2).

Table 5 – Canonical discriminant analysis results on 14 characters of species and morphotypes of *Milla*.
Values and their partial contribution to their canonical functions expressed by standardized coefficients of the discriminant function. [a] Characters with the highest contribution to centroid separation among species.

Variable	can1	can2	can3	can4	can5
Eigenvalue	32.62	7.94	3.03	1.70	1.24
Percentage variation (%)	64.03	79.59	85.53	88.87	91.32
Corm width	0.621	0.189	0.189	0.196	0.015
Leaf length	0.002	0.316	0.209	-0.072	0.235
Leaf width	0.674	0.045	0.287	0.559[a]	0.039
Inflorescence basal axis length	0.897[a]	0.038	0.038	-0.204	0.001
Inflorescence basal axis width	0.535	0.271	0.271	-0.108	0.453[a]
Pedicel length	0.859[a]	0.063	0.063	0.001	0.101
Bract length	0.858[a]	0.630[a]	0.631	-0.157	0.249
Floral tube length	0.097	0.396[a]	0.359	0.053	0.210
Inner tepal width	0.204	0.175	0.593[a]	-0.060	-0.173
Apiculum length	0.206	0.568[a]	0.568	-0.059	-0.152
Anther length	0.886[a]	0.110	0.111	0.095	0.083
Ovary length	-0.322	-0.418	0.634a	0.255	-0.010
Style length	0.171	0.328	0.229	0.297	-0.029
Gynophore length	0.537	-0.384	0.414	0.236	-0.417

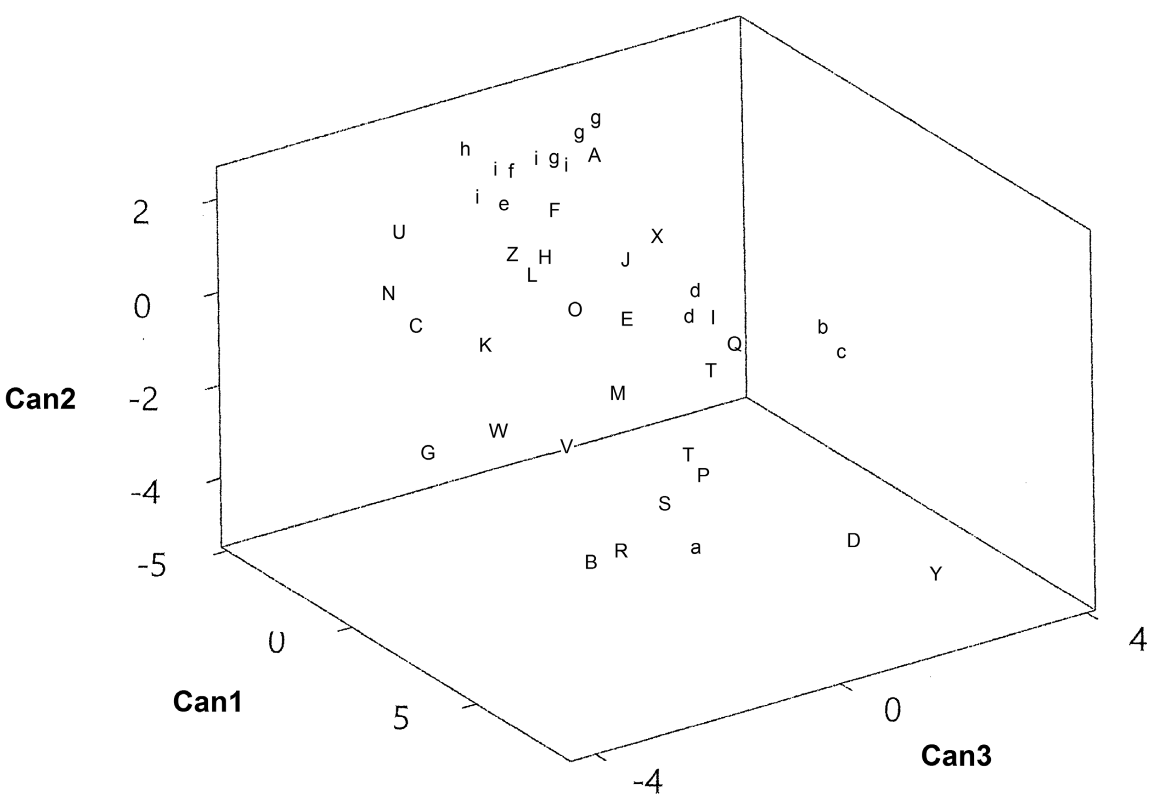

Figure 1 – Graphic representation of the first three canonical functions of the Canonical Discriminant Analysis of 24 variables for eight species of *Milla* and 26 morphotypes of *M. biflora* s. lat. Letters correspond to species and morphotypes, for names see table 3.

Table 6 – Percentage of individuals of *Milla* species and morphotypes that correctly grouped in their group based on the discriminant analysis (DA).

Species and morphotypes	% of individuals
Milla bryanii, *M. magnifica*, *M. mexicana*, *M. rosea*, Morphotype 1 (Oax.2), 3 (BCS), 5 (Zac3), 14 (Oax12), 17 (Oax13), 20 (Oax-Pue2) and 26 (Hgo).	> 80
Milla valliflora, Morphotype 2 (Mor1), 6 (Nay1), 7 (Pue2), 8 (Oax6), 21 (Oax1), 9 (Oax9), 10 (Mich1), 11 (Chis1), 12 (Chis2), 12 (Oax8), 13 (Oax11), 18 (Pue1), 22 (Oax3), 22 (Oax4) and 23 (Zac3).	50–79
Milla biflora (Ags), (Gto), (Mor2), (Pue-Ver), (VM), *M. filifolia*, *M. oaxacana*, *M. potosina*, Morphotype 4 (Oax5), 15 (Oax7), 16 (Oax10), 19 (Gro), 24 (Nay2), 25 (Chih) 25 (Mich2) and 25 (Zac2).	< 49

DISCUSSION

Character variation

Nine out of eighteen quantitative characters contribute the most to differentiate some of the species and morphotypes. Out of these nine characters, six are floral and most of them have not been used before as diagnostic characters for recognizing *Milla* species, except by Ravenna (1971), who used floral tube size for recognizing *M. oaxacana* Ravenna and separating it from *M. biflora*. A unique combination of leaf width, inflorescence basal axis length and diameter, pedicel length, floral bract length, floral tube length, inner tepal width, apiculum length and anther length allows the recognition of seven morphotypes of *Milla* (25%) and four species of *Milla* (see key). However, these quantitative characters do not allow for the separation of the other morphotypes and species of *Milla* (table 6) due to their variation. This is in contrast with the case of other species complexes or genera with a smaller number of species (Pimentel Pereira et al. 2007, Scrivanti et al. 2013, Souza et al. 2014).

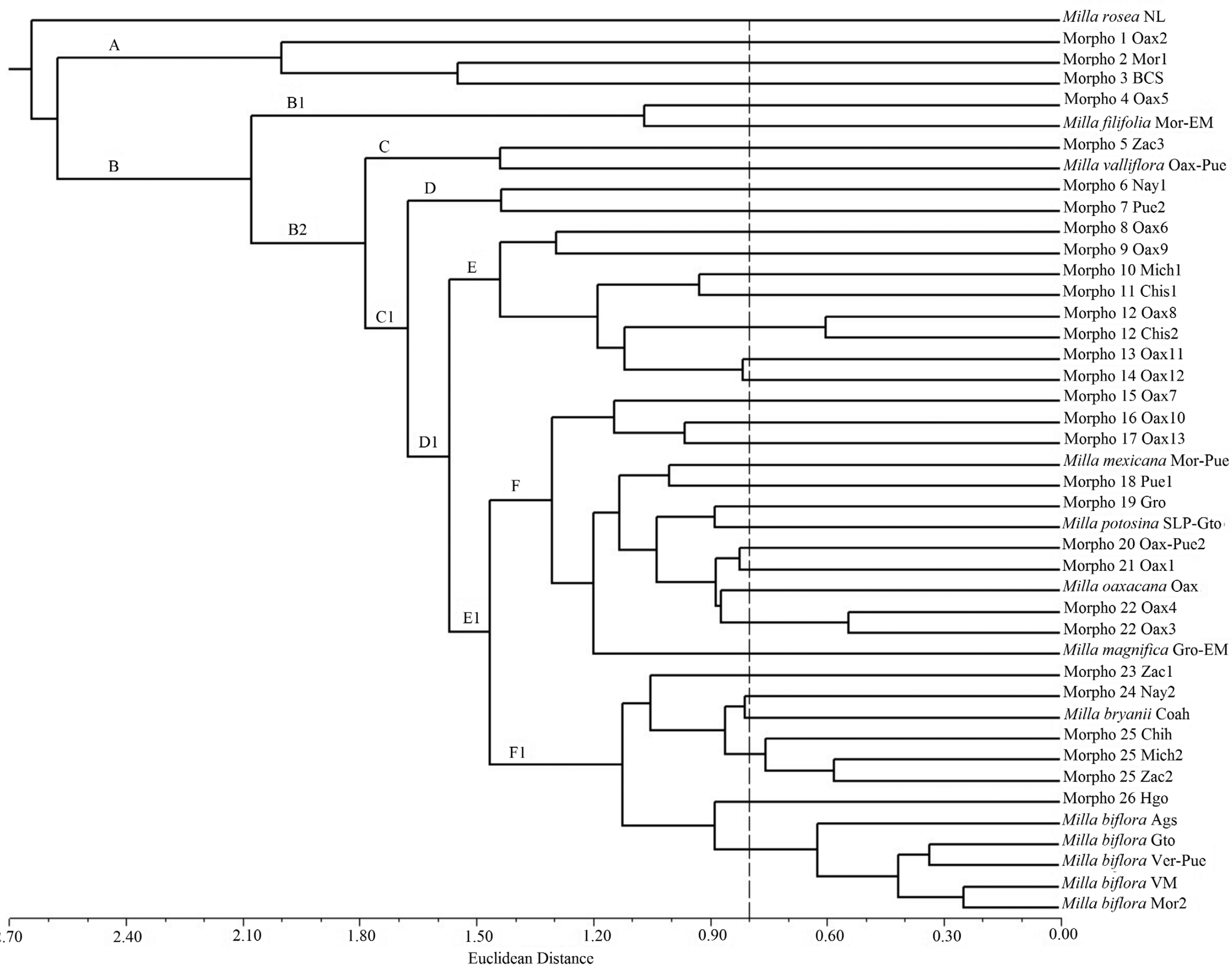

Figure 2 – Cluster analysis for nine species and 26 morphotypes of *Milla* with Euclidean distance coefficient and UPGMA. Euclidean distance for recognizing species is from 0.80 (dotted line).

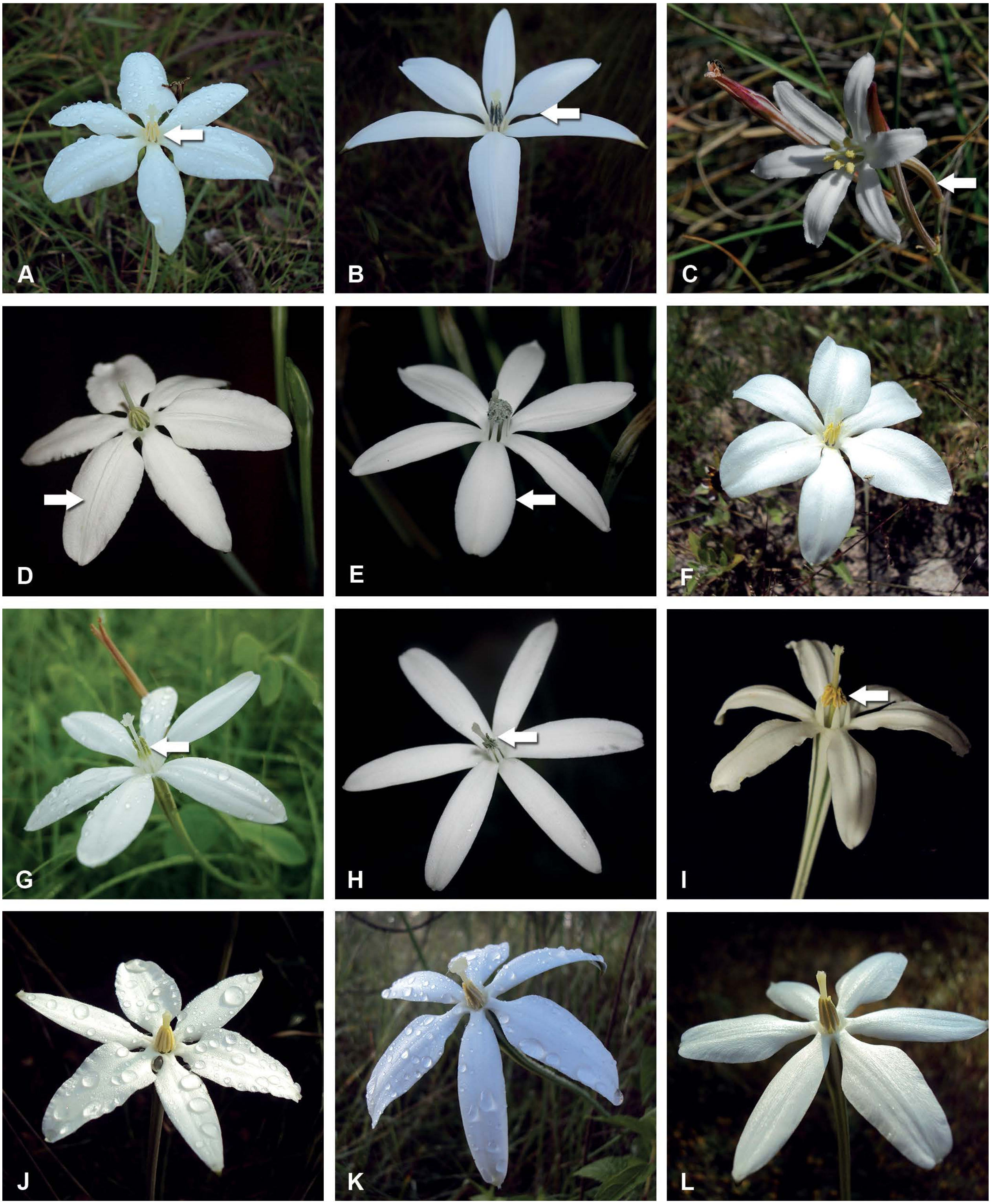

Figure 3 – Floral characters of *Milla* and morphotypes. A, Morphotype 1 (*J. Gutiérrez & al.* 1163) with abruptly attenuated inner tepal base (arrow); B, Morphotype 21 (*J. Gutiérrez & al.* 1160) with attenuated inner tepal base (arrow); C, *Milla rosea* (*J. Gutiérrez & al.* 1218) with floral tube < 5 cm length (arrow); D, *Milla filifolia* (*J. Gutiérrez & I. Escalante* 1133) with inner tepals ovate (arrow); E, *Milla mexicana* (*J. Gutiérrez* 1144) with inner tepals narrowly elliptic (arrow); F, *Milla valliflora* (*J. Gutiérrez & al.* 1151) with solitary flower; G, Morphotype 10 (*J. Gutiérrez & R. Ríos* 1342) with green anthers (arrow); H, Morphotype 19 (*J. Gutiérrez & R. Rios* 1344) with blue anthers (arrow); I, *Milla bryanii* (*J. Gutiérrez & al.* 1226) with yellow anthers (arrow); J, *Milla biflora* (*J. Gutiérrez & al.* 1195) with white flowers that are open during day-time and night-time; K, *Milla biflora* (*J. Gutiérrez & al.* 1362) with elliptic outer tepals, and narrowly elliptic inner ones with widely attenuated at base; L, *Milla biflora* (*J. Gutiérrez & al.* 1254) with filaments < 1 mm in length with yellow anthers.

Key for the *Milla* species and morphotypes of *Milla biflora*

1. Floral tube < 5 cm long, inner tepal < 5.8 mm wide..*M. rosea*
1’. Floral tube > 5 cm long, inner tepal > 5.8 mm wide...2

2. Inner tepal base abruptly attenuated...3
2’. Inner tepal base attenuated or widely attenuated..5

3. Pedicel ≥ 4 cm long, outer floral bract ≥ 1.1 cm, inner tepal ≥ 1.3 cm wide...................Morphotype 1
3’. Pedicel < 3 cm long, outer floral bract < 1 cm, inner tepal < 1.3 cm wide...4

4. Leaf flat, inflorescence basal axis pilose, pedicel 2.5–3 cm long, tepals with 5 nerves...Morphotype 2
4’. Leaf subcircular, inflorescence basal axis smooth, pedicel < 1 cm long, tepals with 3 nerves.............. ....Morphotype 3

5. Floral tube 8–10 cm long, inner tepals ovate..6
5’. Floral tube 10.1–16 cm long, inner tepals narrowly elliptic or elliptic...7

6. Pedicel 1.5–2 cm long conspicuous floral articulation; inner tepal 6.5–7 mm wide.............*M. filifolia*
6’. Pedicel ≥ 3.5 cm long, inconspicuous floral articulation; inner tepal 7.5–8 mm wide.....Morphotype 4

7. Leaves circular, adaxial leaf surface round..8
7’. Leaves subcylindrical, adaxial leaf surface flat...9

8. Leaf denticulations 50–100 µm long, inflorescence basal axis scabrous, flowers 2, pedicel 1–1.5 cm long...Morphotype 5
8’. Leaf denticulations < 50 µm long, inflorescence basal axis smooth, flowers 1, pedicel c. 2.5 cm long. ....*M. valliflora*

9. Inflorescence basal axis smooth; outer floral bract 6–7.4 mm long...10
9’. Inflorescence basal axis scabrous or pilose; outer floral bract 7.5–14 mm long (if inflorescence basal axis smooth, then outer bract > 9 mm long)...11

10. Single flower, anthers yellow, 5 mm long...Morphotype 6
10’. Two flowers in the inflorescence, anthers green, 3.5–3.7 mm long...............................Morphotype 7

11. Leaves in cross section circular or widely depressed. If depressed obovate or narrowly transversely elliptic, then pedicels ≤ 1.8 cm..12
11’. Leaves in cross section narrowly transversally elliptic or widely depressed obovate........................18

12. Floral tube < 12 cm long; pedicel > 2.5 cm long...13
12’. Floral tube > 12 cm long; pedicel < 2.5 cm long...14

13. Leaf denticulations 50–100 µm long; inflorescence basal axis smooth; outer tepals narrowly elliptic, with 5 nerves, anthers green...Morphotype 8
13’. Leaf denticulations < 50 µm long; inflorescence basal axis smooth; outer tepals elliptic, with 7–12 nerves, anthers blue...Morphotype 9

14. Leaves in cross section depressed obovate or narrowly transversally elliptic...................................15
14’. Leaves in cross section widely elliptic or circular..16

15. Leaf denticulations < 50 µm long; inflorescence with more than 2 flowers, tepals with 7–12 nerves.... ....Morphotype 10
15’. Leaf denticulations 50–100 µm long; inflorescence with 2 flowers, tepals with 5 nerves..................... ....Morphotype 11

16. Pedicel 1.5–3 cm long; floral tube 13–14 cm long; anthers 4.5 mm long.....................Morphotype 12
16’. Pedicel < 1.4 cm long; floral tube 10–12.8 cm long; anthers 3.8– 4.2 mm long...............................17

17. Rhizome present; floral tube 12.5–12.8 cm long, outer tepal elliptic............................Morphotype 13
17’. Rhizome absent; floral tube < 12 cm long, outer tepal widely elliptic..........................Morphotype 14

18. Anthers blue or green..19
18’. Anthers yellow..30

19. Inner tepal base widely attenuated...20
19’. Inner tepal base attenuated..22

20. Leaves in cross section transversally elliptic; pedicel < 3 cm; outer bract < 8 mm long; floral tube ≤ 12 cm long, inner tepals 6.5–7 mm wide; anthers 3.5–3.7 mm long..........................Morphotype 15
20’. Leaves in cross section widely depressed obovate; pedicel 4–4.7 cm, outer bract 8–13 mm long; floral tube > 12 cm long, inner tepals 10 mm wide; anthers 4.8 mm long...21

21. Rhizome absent; pedicel < 4 cm long; floral tube ≤ 13 cm long, tepals with 7–12 nerves; anthers blue...
....Morphotype 16
21'. Rhizome present; pedicel > 4 cm long; floral tube > 13 cm long, tepals with 5 nerves; anthers green........
....Morphotype 17
22. Leaf mesophyll compact...23
22'. Leaf mesophyll collapsed...24
23. Rhizome present; leaves in cross section widely depress obovate, adaxial leaf surface round; tepals with 7–12 nerves...*M. mexicana*
23'. Rhizome absent; leaves in cross section obovate depress, adaxial leaf surface flat; tepals with 5 nerves..
....Morphotype 18
24. Leaves smooth, denticulations absent; inflorescence basal axis smooth; inner tepals > 1 cm wide..........
....*M. magnifica*
24'. Leaves striated, denticulations present; inflorescence basal axis scabrous or pilose, inner tepals ≤ 1 cm wide...25
25. Pedicel ≤ 3 cm long; floral tube 9–11 cm long, inner tepal 7–8.5 mm wide.......................................26
25'. Pedicel 3.5–5 cm long; floral tube 12–16 cm long, inner tepal > 12 mm wide.................................27
26. Rhizome absent; floral tube ≤ 10 cm long, inner tepal 8–8.5 mm wide, tepals with 3 nerves...............
....*M. potosina*
26'. Rhizome present; floral tube > 10 cm long, inner tepal 7 mm wide, tepals with 5 nerves.........
....Morphotype 19
27. Vascular bundles in leaves 7–12...28
27'. Vascular bundles in leaves > 12...29
28. Rhizome present; leaf prominences short; tepals elliptic; anthers green........................Morphotype 20
28'. Rhizome absent; leaf prominences long; tepals narrowly elliptic; anthers blue.............Morphotype 21
29. Fibres of foliar mesophyll with thin walls; pedicel ≥ 4.6 cm long; bract < 8.5 mm long; floral tube 12–12.4 cm long, inner tepals elliptic; flower opening nocturnal only..............................*M. oaxacana*
29'. Fibres of foliar mesophyll with thick walls; pedicel 3.5–4.6 cm long; bract 10–12 mm long; floral tube 12.5–13.5 cm long, inner tepals narrowly elliptic; flower opening diurnal-nocturnal.....Morphotype 22
30. Floral tube < 13.5 cm long...31
30'. Floral tube > 13.5 cm long...34
31. Leaves smooth, lamina denticulations absent...Morphotype 23
31'. Leaves striated, lamina denticulations present...32
32. Vascular bundles in leaf > 12; outer floral bract 6–10 mm long; inflorescence with 2 flowers; inner tepals 5.5–10 mm wide...33
32'. Vascular bundles in leaf 7–12; outer floral bract > 10 mm, inflorescence with 3 or more flowers; inner tepal > 10 mm wide...Morphotype 25
33. Fibres of foliar mesophyll with thick walls, foliar denticulations short; floral tube articulation inconspicuous, pedicel c. 2.5 cm long; anthers 4.5 mm long...Morphotype 24
33'. Fibres of foliar mesophyll with thin walls, foliar denticulations long; floral tube articulation conspicuous, pedicel up to 3.5 cm long; anthers 2.6 mm long...*M. bryanii*
34. Inflorescence basal axis scabrous, pedicel > 1 cm long...*M. biflora* s. str.
34'. Inflorescence basal axis smooth; pedicel ≤ 1 cm long..Morphotype 26

Cluster analysis

The combination of qualitative and quantitative characters favoured the separation of 26 morphotypes and species of *Milla* to a cut-off level ≥ 0.80 of dissimilarity. Qualitative characters such as the occurrence of rhizomes, leaf denticulations, pubescence of the inflorescence basal axis and leaves, type of pedicel articulation, time of flower opening, tepal shape base and number of nerves, allowed Howard (1999) to separate and describe *M. mexicana* T.M.Howard and *M. potosina* T.M.Howard. Our analysis supports the assertions of Howard (1999), who suggested that qualitative characters in *Milla* have a higher diagnostic value.

Our analysis also revealed that floral tube size is important for recognizing the core groups in *Milla* (fig. 2). *Milla rosea* is the most dissimilar species of the genus because of its short floral tube (< 5 cm long) and inner tepal (< 5.8 mm wide; fig. 3C). As mentioned, groups A and B, and subgroups B1 and B2 were also recognized by floral tube size and basal inner tepal shape. For example, a floral tube size of 8–10 cm long and ovate inner tepals distinguish subgroup B1, containing *M. filifolia* T.M.Howard and morphotype 4 (fig. 3D). However, *M. filifolia* differs from morphotype 4 by the presence of articulate pedicels 1.5–2 cm long and inner tepals 6.5–7 mm wide.

Group B2 showed the longest floral tubes (10–16 cm) and narrowly elliptic or elliptic inner tepals (fig. 3E). In this subgroup, *M. valliflora* J.Gut. & E.Solano along with morphotype 5 (Zac3) form group C, by showing a circular leaf shape in cross section and having one or two flowers, but *M. valliflora* is distinguished by having short leaf denticulations, inflorescence basal axis smooth, a solitary flower and a pedicel of almost 2.5 cm long (fig. 3F). Group C1 has subcylindrical leaves, a flat adaxial leaf surface and two or more flowers, as well as a smooth inflorescence basal axis and an outer floral bract 6–7.4 mm long. Groups D and D1 are recognized by the presence of denticulations and trichomes in the inflorescence basal axis and by the size of the floral bract. Widely elliptic or circular leaf shape, in cross section, separates group E, which included morphotypes from Chiapas, Oaxaca and morphotype 10 from Michoacán (fig. 3G). Blue or green anthers support group F with species *M. magnifica*, *M. oaxacana*, *M. mexicana*, *M. potosina* and nine morphotypes distributed in southern Mexico (Fig. 3H). Yellow anthers are present in group F1, which recovers *M. biflora* s. str. and *M. bryanii* and morphotypes with a geographical distribution restricted to the Trans-Mexican Volcanic Belt, the Mexican Plateau and the Sierra Madre Occidental (fig. 3I–L).

The similarity of *M. bryanii* with morphotype 24 (Nay2) is explained by the presence of striate leaves with foliar denticulations and floral tube > 10 cm long. *Milla magnifica* has smooth leaves with collapsed mesophyll, smooth inflorescence basal axis and inner tepals > 1 cm wide. In contrast, *M. mexicana* shows leaves with a compacted mesophyll, the same as morphotype 18 (Pue2). *Milla oaxacana* and morphotype 22 (Oax3 and Oax4) have more than twelve vascular bundles in the leaf mesophyll and a floral tube ≤ 13.5 cm long (fig. 3B, E & H). *Milla potosina* and morphotype 19 (Gro) show pedicels ≤ 3 cm long, floral tube 9–11 cm long and inner tepals 7–8.5 mm wide. *Milla rosea* has tepals with a pink colour in their outer surface (fig. 3C), floral tube < 5 cm long and inner tepal < 5.8 mm wide – this combination supports its delimitation.

The results of this study allow the recognition of 26 morphotypes differing from the nine evaluated species, including *M. biflora* s. str. The unique combination of characters of each species is diagnostic for its identification (see key). Cluster analysis is congruent with the Gándara et al. (2014) hypothesis, who based on a molecular data (chloroplast and nuclear) suggested that under the name *M. biflora* other taxa are to be recognized.

Milla biflora s. str.

The cluster analysis revealed that the eight *Milla* species contrasted against *M. biflora* have a dissimilarity value above 0.80 in the Euclidean distance. In the particular case of *M. biflora* (VM), this population plus Ags, Gto, Pue-Ver and morphotype 2 populations have a dissimilarity value below 0.80 (fig. 2). Based on this value, we propose to recognize those populations together with the one from the Mexican Basin as a single entity, *M. biflora* s. str. The precise geographic location based on the examination of the type specimen of *M. biflora* deposited in the Real Jardín Botánico (Madrid) (McVaugh 1977), allowed the evaluation of the quantitative and qualitative characters used in this study, such as: fistulose leaves, pedicel 1.5–3 cm in length lacking an evident articulation, white flowers that are open during the day and night, three tepal nerves, elliptic outer tepals, narrowly elliptic inner tepals with widely attenuated base, filaments less than 1 mm long and yellow anthers (fig. 3J, K & L). These characters are corroborated in the materials collected in the Mexican Basin and were the base for its morphological recognition. Qualitative characters that were also observed in the type specimen and corroborated in herbarium materials and in plants examined in the field support *M. biflora* s. str. distinction from the morphotypes distributed in North and Western Mexico, the Rio Balsas Basin and Sierra Madre del Sur. The 26 morphotypes here segregated of *M. biflora* are congruent with molecular phylogeny (Gutiérrez et al. submitted) and they need to be formally named and described.

CONCLUSIONS

Multivariate analyses identified nine quantitative characters that, taken together with qualitative characters, allowed to recognize nine species of *Milla* including *M. biflora* s. str. and to separate 26 morphotypes of *M. biflora* s. str. as well. The new delimitation of *M. biflora* s. str. proposed herein is based on a unique combination of characters and on its distribution confined to the south of the Mexican Plateau and central and east part of the Trans-Mexican Volcanic Belt. Moreover, the species of *Milla* have a combination of qualitative and quantitative characters that allows proposing an identification key.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of a list of selected specimens included in the analyses.

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REFERENCES

Angiosperm Phylogeny Group (2016) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Botanical Journal of the Linnean Society 181: 1–20. https://doi.org/10.1111/boj.12385

Arroyo-Cosultchi G., Terrazas T., Arias S., López-Mata L. (2010) Delimitación de Neobuxbaumia mezcalaensis y N. multiareolata (Cactaceae) con base en análisis multivariados. Boletín de la Sociedad Botánica de México 86: 53–64.

Birch A.N.E., Tithecott M.T., Bisby F.A. (1985) Vicia johannis and wild relatives of the faba bean: a taxonomic study. Economic Botany 39: 177–190.

Cavanilles A.J. (1793) Icones et descriptiones plantarum. Vol. 2. Madrid, Royal Printing Office.

Chung M.G., Kang S.S. (1994) Morphometric analysis of the genus Hemerocallis L. (Liliaceae) in Korea. Journal of Plant Research 107: 165–175. https://doi.org/10.1007/BF02346013

Ferreira Da Costa A., Pena-Rodrígues P.J.F., Lapa-Wanderley M.D.G. (2009) Morphometric analysis and taxonomic revision of the Vriesea paraibica complex (Bromeliaceae). Botanical Journal of the Linnean Society 159: 163–181. https://doi.org/10.1111/j.1095-8339.2008.00919.x

Galván R. (2001) Alliaceae. In: Calderón de Rzedowski G., Rzedowski J. (eds) Flora Fanerogámica del Valle de México: 1252–1254. Mexico City, Instituto de Ecología, A.C., Comisión Nacional para el Conocimiento y Uso de la Biodiversidad.

Gándara E., Specht C.D., Sosa V. (2014) Origin and diversification of the Milla clade (Brodiaeoideae, Asparagaceae): a Neotropical group of six geophytic genera. Molecular Phylogenetics and Evolution 75: 118–125. https://doi.org/10.1016/j.ympev.2014.02.014

Gutiérrez J., Solano E. (2015) Milla valliflora (Themidaceae), a new species from southern Mexico. Brittonia 67: 43–47. https://doi.org/10.1007/s12228-014-9354-0

Gutiérrez J., Salgado J., Grego-Valencia D., Terrazas T. (2015) Morfología y anatomía foliar de los géneros del complejo Milla (Themidaceae). Revista Mexicana de Biodiversidad 86: 652–660. https://doi.org/10.1016/j.rmb.2015.06.008

Gutiérrez J., Terrazas T., Luna-Vega I. Salazar G. (submitted) Phylogenetic analyses of the Milla complex (Brodiaeoideae: Asparagaceae), with emphasis on Milla. Botanical Journal of the Linnean Society.

Hejazi J., Bahrami A.R., Vaezi J., Memariani F., Joharchi M.R. (2012) Application morphometric and taxonomic study of the genus Carex L. (Cyperaceae) in Northeast of Iran. Journal of Biology and Today's World 1: 63–81. https://doi.org/10.15412/J.JBTW.01010105

Henderson A., Ferreira E. (2002) A morphometric study of Synechanthus (Palmae). Systematic Botany 27: 693–702.

Henderson A.J. (2004) A multivariate analysis of Hyospathe (Palmae). American Journal of Botany 91: 953–965. https://doi.org/10.3732/ajb.91.6.953

Hernández-Hernández V., Terrazas T., Delgadillo-Moya C. (2009) The Dryopteris patula complex (Dryopteridaceae) in Mexico: morphometric analyses. Boletín de la Sociedad Botánica de México 85: 103–112.

Howard T.M. (1999) Three new Milla species from Mexico. Herbertia 54: 232–237.

Jakovljević K., Šinžar-Sekulić J., Vukojičić S., Kuzmanović N., Lakušić D. (2013) Leaf anatomy of Carex humilis (Cyperaceae) from Central and South Eastern Europe. Botanica Serbica 37: 3–11.

Knowles G.B., Westcott F. (1839) Loudon's Hortus Britannicus: a catalogue of all the plants indigenous, cultivated in, or introduced to Britain. Part I. The Linnaean arrangement: part II. The Jussieuean arrangement. 3rd Ed. London, J.C. Loudon. https://doi.org/10.5962/bhl.title.68944

Levin G.A. (1998) Evolution in the Acalypha gracilens/monococca complex (Euphorbiaceae): morphological analysis. Systematic Botany 23: 269–287. https://doi.org/10.2307/2419505

Martínez-Cabrera D., Zavala-Chávez F., Terrazas T. (2011) Estudio morfométrico de Quercus sartorii y Q. xalapensis (Fagaceae). Revista Mexicana de Biodiversidad 82: 551–568.

McVaugh R. (1977) Botanical results of the Sessé & Mociño Expedition (1787–1803). Contributions from the University of Michigan Herbarium. Ann Arbor, Hunt Institute for Botanical Documentation, Carnegie Mellon University.

Moore H.E. (1953) The genus Milla (Amaryllidaceae-Allieae) and its allies. Gentes Herbarum 8: 262–294.

Nees von Esenbeck C.G., Schauer S. (1847) Enumeratio et descriptiones generum novorum specierumque plantarum in terries Mexicanis crescentum, quas Ruhlandus, Coloniensis, de Berghes et Dr. Aschenborn collegerunt. Linnaea 19: 681–734.

Pimentel Pereira M., Estévez-Pérez G., Sahuquillo Balbuena E. (2007) European sweet vernal grasses (Anthoxanthum: Poaceae, Pooideae, Aveneae): a morphometric taxonomical approach. Systematic Botany 32: 43–59. https://doi.org/10.1600/036364407780360201

Rafinesque C.S. (1836) Flora Telluriana. 2: 12. 1837 [1836 publ. Jan–Mar 1837]. Philadelphia, H. Probasco. https://doi.org/10.5962/bhl.title.7751

Ravenna P.F. (1971) A new Milla species from Mexico. Plant Life 27: 60, 89–90.

Rolhf F.J. (2000) NTSYS. Numerical Taxonomy and Multivariate Analysis System. Version 2.1. New York, Applied Biostatistics Inc.

Sánchez D., Arias S., Terrazas T. (2013) Análisis morfométrico de las especies de Echinocereus sección Triglochidiati (Cactaceae) en México. Brittonia 65: 368–385. https://doi.org/10.1007/s12228-012-9285-6

SAS Institute (2008) SAS/STAT User's Guide, Release 9.03 Edition. Cary, SAS Institute Inc.

Scrivanti L.R., Norrmann G.A., Anton A.M. (2013) Delimiting species boundaries within the Bothriochloa saccharoides complex (Poaceae) through morphometric analysis. Phytotaxa 89: 24–42. https://doi.org/10.11646/phytotaxa.89.1.2

Sneath P.H., Sokal R.R. (1973) Numerical taxonomy. The principles and practices of numerical classification. San Francisco, W.H. Freeman and Company.

Souza I.M., Funch L.S., de Queiroz L.P. (2014) Morphological analyses suggest a new taxonomic circumscription for Hymenaea courbaril L. (Leguminosae, Caesalpinioideae). PhytoKeys 38: 101–118. https://doi.org/10.3897/phytokeys.38.7408

Thompson S.W., Lammers T.G. (1997) Phenetic analysis of morphological variation in the Lobelia cardinalis complex (Campanulaceae:Lobelioideae). Systematic Botany 22: 315–331. https://doi.org/10.2307/2419460

van den Berg R.G., Miller J.T., Ugarte M.L., Kardolus J.P., Villand J., Nienhuis J., Spooner D.M. (1998) Collapse of morphological species in the wild potato Solanum brevicaule complex (Solanaceae: sect. Petota). American Journal of Botany 85: 92–109.

Willdenow C.L. (1799) Species Plantarum, exhibentes plantas rite cognitas ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas. Vol. II(1). Berlin, G.C. Nauk.

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