SPECIATION AND POPULATION GENETICS

Polyploidy, the major speciation mechanism in *Muscari* subgenus *Botryanthus* in the Iberian Peninsula

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Currently, three species of *Muscari* subg. *Botryanthus* are recognized in the Iberian Peninsula: two diploids ($2n = 18$), *M. atlanticum* and *M. cazorlanum*, and one morphologically variable species with three different ploidy levels, *M. neglectum* ($2n = 36, 45, 54$). We have made a comparative study of numerous Iberian populations to clarify the taxonomy and evolution of this group. To this end we carried out morphological and cytogenetic analyses, and phylogenetic analysis of the internal transcribed spacers of nuclear ribosomal DNA. Comparative and UPGMA analyses of the morphological characteristics show that the different ploidy levels of *M. neglectum* represent different species. We describe the pentaploid and hexaploid levels as two new species, *M. olivetorum* ($2n = 45$) and *M. baeticum* ($2n = 54$), each with an exclusive combination of morphological characters and a characteristic ecological behavior pattern. Phylogenetic study of ITS shows that the two new species are not autopolyploids from *M. neglectum* but allopolyploids. These findings are supported by the additivity of the three ITS variants found in *M. olivetorum* with the ITS of *M. neglectum* and *M. baeticum*, and also by morphology. Possible parents for both new species are proposed. Absence of homogenization between homeologous *M. olivetorum* nrDNA loci is explained by the absence of sexual reproduction and by nucleolar dominance, indicating that this is a recent species.

KEYWORDS: cytogenetic analysis, Iberian Peninsula, ITS phylogeny, morphological analysis, *Muscari*, polyploidy, speciation

INTRODUCTION

The genus *Muscari* Mill. consists of approximately 35 to 55 bulbous species grouped into four subgenera (*Botryanthus* (Kunth) Zahar., *Leopoldia* (Parl.) Zahar., *Muscari, Pseudomuscari* Stuart) within a distribution area extending from the Macaronesian region to the Caucasus, although being mainly found in the Mediterranean basin (Karlén, 1991). Numerous karyological studies of *Muscari* have been made since the middle of the last century, and these, combined with morphological evidence, have provided most of the taxonomic data on the genus (Garbari, 1966, 1972, 1973; Bentzer, 1972a; Ruiz-Rejón, 1978; Ruiz-Rejón & Oliver, 1978; Karlén, 1984a, b; Ruiz-Rejón & al., 1985; 1986). In addition to the contribution of karyology to the systematics of *Muscari*, these studies also showed that the major evolutionary mechanisms involved in the diversification of the genus are chromosome rearrangement and polyploidy (Bentzer, 1969, 1972b, 1974; Bentzer & Ellmer, 1975; Oliver & Ruiz-Rejón 1980; Ruiz-Rejón & al., 1985; Valdés & Díaz-Lifante, 1992; Cuñado & al., 2000; De la Herrán & al., 2001).

Polyploidy is one of the main evolutionary mechanisms promoting genetic diversity and speciation in plants (Stebbins, 1950; Grant, 1971). Polyploids generally differ markedly from their progenitors in morphological, ecological, physiological and cytological characteristics that can contribute both to exploitation of a new niche and to reproductive isolation (cf. Ramsey & Schemske, 2002). However, we know relatively little about how neopolyploids originate and establish themselves in populations. Theory suggests that the probability of polyploid formation is limited by the rate of non-reduced gamete production and by the pair patterns of these gametes, while the probability of establishment is limited both by the difficulty to find individuals of the same cytotype to breed with, and by hybrid non-viability (Levin, 1975; Fowler & Levin, 1984; Ramsey & Schemske, 1998). Stebbins (1950) demonstrated that polyploidy is more frequent in perennial plants than in annuals (having more time to breed with the same cytotype), in vegetative or apomictic reproduction systems (which produce more individuals of the same cytotype to breed with, or do not even require cross-reproduction), and in self-fertilized species.

Within *Muscari*, polyploidy is a frequent phenomenon in the subgenus *Botryanthus*, in which many species have a range of different ploidy levels (Davis & Stuart, 1984; Karlén, 1984b). This is specially true for the highly polymorphic *M. neglectum* Guss. complex (Valdés & Mejías, 1988; Karlén, 1991; Valdés, 1996; Garbari, 2003) which shows ploidy levels ranging from diploid in Greek and Turkish populations to octoploid in Greece (with all the levels inbetween; cf. Garbari, 2003). Many extreme forms of *M. neglectum* have therefore been recognized as separate species (Karlén, 1984a, 1991).

In the Iberian Peninsula tetraploid ($2n = 4x = 36$), pentaploid ($2n = 5x = 45$) and hexaploid ($2n = 6x = 54$) cytotypes have been described in *M. neglectum* (Valdés, 1970; Barros-Neves, 1973; Löve & Kjellqvist, 1973; Sañudo & Ruiz-Rejón, 1975; Ruiz-Rejón, 1976; Ruiz-Rejón & Oliver, 1978; Marco-Moll & Notivol-Tejero, 1979; Ruiz-Rejón & al., 1986). Besides *Muscari neglectum*, in the Iberian Peninsula there are two other recognized species of the subgenus *Botryanthus*: *M. atlanticum* Boiss. & Reut., and *M. cazorlanum* Soriano & al. The first is restricted to a few populations in the south and east of the Iberian Peninsula and in Morocco. It grows in narrow cracks in rocks and in stony places among shrubs on limestone substrates (cf. Valdés, 1996). For a long time *M. atlanticum* was considered a synonym for *M. neglectum* (incl. *M. racemosum*). Ruiz-Rejón & al. (1986) however considered it a diploid species ($2n = 18$) with the diagnostic morphological characters indicated by Boissier & Reuter (1852).

Muscari cazorlanum only occurs in the Sierra de Cazorla mountains (province of Jaén in southern Spain). It is morphologically very close to *M. atlanticum*, and differs mainly in the colour of the fertile flowers (greenish in *M. cazorlanum* and bluish-violet or bluish-purple in *M. atlanticum*). *Muscari cazorlanum* is also a diploid species ($2n = 18$) (Soriano & al., 1990).

Bearing in mind that there are four different ploidy levels and at least three different species of *Muscari* subg. *Botryanthus* in the Iberian Peninsula, we have attempted to clarify the taxonomy and evolution of this group of species. To this end, we performed morphological, ecological and cytogenetic analyses, and phylogenetic analysis of the internal transcribed spacers (ITS) of the nuclear ribosomal DNA (nrDNA), on numerous populations of this subgenus in the south and east of the Iberian Peninsula (from the province of Cádiz in the west to the province of Valencia in the east).

MATERIALS AND METHODS

Plant material. — Species used in this study include the three species of *Muscari* subg. *Botryanthus* currently recognized in the Iberian Peninsula (*M. atlanticum*, *M. cazorlanum*, *M. neglectum*) and the two new species proposed here (*M. olivetorum*, *M. baeticum*) (Appendix 1). The two Spanish species of *Muscari* subg. *Leopoldia* (*M. comosum* (L.) Mill. and *M. matritensis* Ruiz-Rejón & al.) were used as outgroups in the molecular analysis.

Morphology. — A comparative analysis of morphological characteristics was performed on 21 Spanish populations of *Muscari* subg. *Botryanthus* (Appendix 1). Characters in the morphological analysis (quantitative and qualitative) were those with the greatest variation, and at least 30 measurements were taken in each population. Flower characters were measured during anthesis of middle-inflorescence flowers. Figure 1 illustrates inflorescence characters and the location of the anthers in relation to the apex of the perigone.

A data matrix was obtained from 19 morphological characters and subjected to a classification analysis. The 11 populations in which it was possible to measure all 19 characters were used. In order to construct the data matrix, the average value for each continuous-quantitative character was calculated. A logarithmic transformation of these average values was made [$\log (x + 1)$]. The nine qualitative characters were constant within the different populations. The classification method used was UPGMA (Unweighted Pair-Groups Method using Arithmetic averages), using a dissimilarity matrix generated with the Euclidean distances. The statistical program used was SPSS version 12.0.

Karyological analysis. — Chromosome numbers were counted at metaphase in bulb roots. Roots were pretreated with 8-hydroxy-quinoleine, fixed in ethyl alcohol–acetic acid (3 : 1), hydrolysed in 1 N HCl, stained in acetic orcein solution, and then flattened for light microscopy (Darlington & La Cour, 1969).

Molecular analysis. — In this analysis the ITS (ITS-1, 5.8S, ITS-2) region of the nrDNA was used as molecular marker. Intraspecific variability was explored by sequencing five different clones in each species (only one for outgroups), giving 27 ITS sequences in total. The sequence obtained from each clone was identified with the name of the species together the clone number. EMBL accession numbers are shown in Appendix 1.

Total genomic DNA was extracted, using the CTAB method (Doyle & Doyle, 1987), from fresh leaves collected in the wild. The entire ITS region (ITS-1, 5.8S, ITS-2) was amplified by PCR, using primers N-nc18s10 and C26A (Wen & Zimmer, 1996). PCR reactions were performed in a volume of 50 µl under standard conditions (Innis & al., 1990).

PCR products were ligated into the pGEM-Teasy Vector System (Promega) and cloned in JM109 cells following the manufacturer's recommendations (Promega).

Automated sequencing of the purified plasmid clones was performed in both directions using the ge-

neric primers T7 and SP6. Thermal-cycling reactions were performed using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing was then carried out on a 3100-Avant Genetic Analyzer. Nucleotide sequences were edited with the SEQMAN II v. 3.61 program from the DNAstar software package (LASERGEN). BLAST searches confirmed that our products belonged to the ITS region of *Muscari*, based on their strong identity with the ITS region of members of other families of Asparagales (Themidaceae and Agavaceae) and also because of the identity of the end of their 18S gene with several species of Hyacinthaceae. These comparisons also allowed us to establish the boundaries of the spacer regions.

Multiple ITS sequences from all taxa were aligned using the CLUSTAL option of the MEGALIN program from the DNAstar software package (LASERGEN), and then a visual inspection was carried out. We calculated the average length and the G + C content of the sequences, and the divergence (*p*-distance) between them using PAUP* version 4.0b10 (Swofford, 2003).

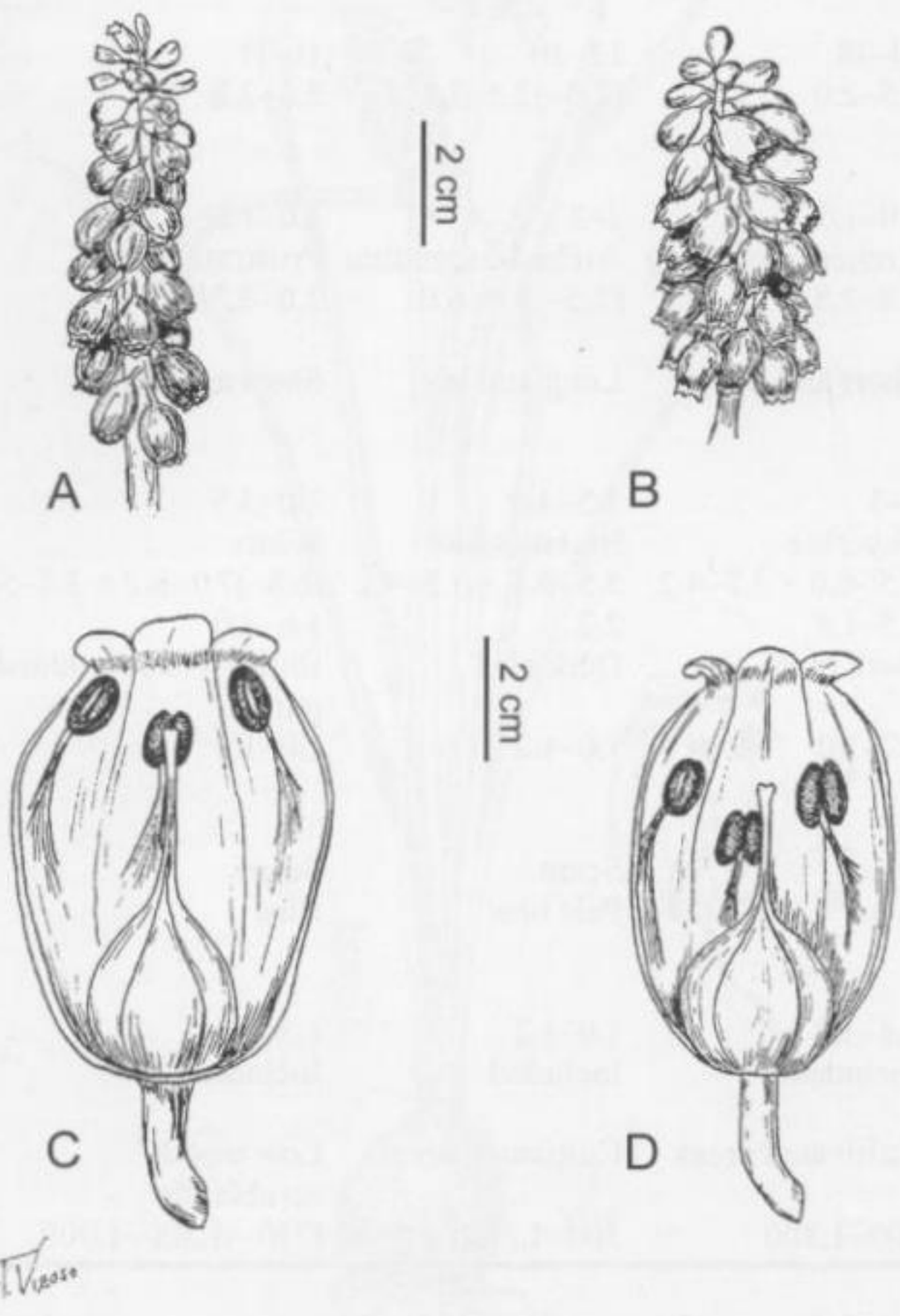

Fig. 1. Inflorescence types and location of the anthers in relation to the apex of the perigone. A, inflorescence long and lax; B, inflorescence short and dense; C, anthers exserted; D, anthers included.

Phylogenetic analyses were performed using two optimality criteria: maximum parsimony (MP) and maximum likelihood (ML), as implemented in PAUP* 4.0b10. Parsimony analysis involved heuristic searches. The data matrix was subjected to 1,000 replicates of random sequence additions using tree bisection-reconnection (TBR) branch-swapping under the Fitch criterion (unordered states and equal weights). Gaps were treated as missing data. Only ten trees were allowed to be held at each step, in order to minimize the time the algorithms spent searching for trees on sub-optimal islands. The starting tree was obtained by stepwise addition. The characters were optimized by accelerated transformation. Finally, 1,000 bootstrap replicates (BS: Felsenstein, 1985) with 10 heuristic searches were performed to assess internal support for nodes. Descriptive statistics reflecting the amount of phylogenetic signal in the parsimony analysis were provided by the consistency index (CI: Kluge & Farris, 1969), the retention index (RI: Swofford, 1993), and by the homoplasy index (HI: Swofford, 1993).

ML searches were implemented using the best-fit nucleotide substitution model for the ITS data. This model was selected using Modeltest 3.06 (Posada & Crandall, 1998), and the starting tree was the most parsimonious tree obtained in the parsimony analysis. Bootstrap values were calculated using 1,000 pseudoreplicates and TBR branch-swapping which began with the topology obtained from the Neighbor-Joining analysis.

RESULTS

Morphology. — The main morphological characters used to differentiate the Spanish species of *Muscari* subg. *Botryanthus* are shown in Table 1. The main differences between the diploid species, *M. cazorlanum* and *M. atlanticum*, and the polyploids is that the diploids have numerous sterile flowers (up to 50% of all flowers) and exserted anthers. The diploids and *M. baeticum* produce a maximum of two bulbils, while the diploids have long, lax inflorescences and pale-blue sterile flowers, in common with *M. olivetorum*. *Muscari cazorlanum* is clearly distinguishable from all other species by having greenish fertile flowers, which turn slightly violet only at the end of flowering.

One of the main differences between the polyploid species is that *M. baeticum* produces very few bulbils (0–2), whereas *M. neglectum* and *M. olivetorum* produce 10 to 20 (50) bulbils for each bulb. Furthermore, *M. baeticum* shows bluish-violet or bluish-purple fertile flowers and prostrate leaves, while *M. neglectum* and *M. olivetorum* have dark-blue fertile flowers and arched-ascending leaves. *Muscari olivetorum* differs from *M. neglectum*, however, in the more robust plant, with wider bulb, scape and leaves, and longer anthers. *Muscari olivetorum* also

has long, lax inflorescences, while in *M. neglectum* the inflorescence is short and dense (see Figs. 2, 3). After studying the type specimen (lectotypus) of *M. neglectum* Guss., we conclude that it belongs to the tetraploid cytotype on the base of the following morphological features: (1) numerous bulbils (only 0–2 bulbils in diploid and hexaploid species; Table 1); (2) very thin scapes and leaves (width: 1.5–2.0 mm and 1.8–2.5 mm respectively), short anthers (0.8–1.0 mm), and short and dense inflorescences. These characteristics agree with the features of the tetraploid species and reject the pentaploid species as the type specimen (Table 1). All these evidences thus support *M. neglectum* as the tetraploid species.

UPGMA analysis yielded a phenogram in which the different populations were grouped together according to their taxonomic affinity. This phenogram supported the morphological separation of *M. olivetorum* and *M. baeticum* (Fig. 4). The branching diagram shows the primary separation of the diploid species from the remainder species followed by *M. baeticum* and the other polyploids. *Muscari olivetorum* populations are phenetically more closely related to *M. neglectum* than to *M. baeticum*.

Chromosome counts. — Of the 21 populations studied, the diploid chromosome level ($2n = 18$) was confirmed for seven populations (one of *M. cazorlanum*

Table 1. Principal morphological characters and habitat of the Iberian species of *Muscari* subg. *Botryanthus*.

Character	*M. cazorlanum* ($2n = 18$)	*M. atlanticum* ($2n = 18$)	*M. neglectum* ($2n = 36$)	*M. olivetorum* ($2n = 45$)	*M. baeticum* ($2n = 54$)
Bulb					
Width (cm)	1.5–2.5	(1.5–)1.8–2.4	1.2–1.7	1.5–2.5	1.2–1.6
Colour	Light chestnut to dark brown	Dark brown to black	Straw to brownish	Light straw	Dark brown
Bulbils[a]	0–2	0–1	Numerous	Numerous	0–2
Scape					
Length (cm)	12–25	12–22	14–18	13–20	11–17
Width (mm)	2–3	2–3	1.5–2.0	(2.0–)2.5–3.5	2.0–2.8
Leaves					
LL/SL[b]	1.0–1.5	0.75–1.00	1.0–1.5	1–2	1.0–1.5(–2.0)
Direction	Arched-ascending	Arched-ascending	Arched-ascending	Arched-ascending	Prostrate
Width (mm)	2–4	2.0–3.5	1.8–2.5	(2.5–)3.0–6.0	2.0–2.7
Inflorescence	Long and lax	Long and lax	Short and dense	Long and lax	Short and dense
Fertile flowers					
Pedicels (mm)	3.5–5.0	2.5–4.5	2–3	3.5–4.0	3.0–3.5
Bracts	White	White	Sky-blue	Bluish-white	White
Size (mm)	4–6 × 3–4	5.5–8.0 × 3.5–5.5	5.5–6.0 × 3.7–4.2	5.5–6.5 × 3.5–4.2	(6.8–)7.0–8.2 × 3.5–5.0
Mouth diameter (mm)	2.0–2.5	2–3	1.5–1.8	2.2	1.6–3.0
Colour	Greenish	Bluish-violet or bluish-purple	Dark blue	Dark blue	Bluish-violet or bluish-purple
Lobes (mm)	1	1	0.7–1.0	1.0–1.2	1.0–1.5
Sterile flowers[c]					
Quantity	Numerous	Numerous	Scant	Scant	Scant
Colour	Pale blue	Pale blue	Blue	Pale blue	Blue
Anthers[d]					
Length (mm)	1.0–1.2	1.0–1.2	0.8–1.0	1.0–1.2	1.3–1.5
Position	Exserted	Exserted	Included	Included	Included
Habitat	Low woody scrublands	Low woody scrublands	Cultivated areas	Cultivated areas	Low woody scrublands
Altitude (m a.s.l.)	500–900(–1,200)	100–700(–1,200)	100–1,300	300–1,300	(750–)1,300–1,900

[a]Bulbils numerous: > 10.

[b]LL/SL: ratio leaf length/scape length.

[c]Sterile flowers scant: < 20% in total; numerous: ≥ 20% in total.

[d]Figure 1 illustrates inflorescence and location of anther characters.

and six of *M. atlanticum*), the tetraploid level ($2n = 36$) for four populations (*M. neglectum*), the pentaploid level ($2n = 45$) for five (*M. olivetorum*) and the hexaploid level ($2n = 54$) for five (*M. baeticum*) (Table 2). Fig. 5 shows a metaphase plate of each species.

Molecular analysis. — Alignment of all 27 ITS sequences resulted in a 644 bp matrix. Table 3 shows the main characteristics of these sequences. Of the 15 gaps introduced, two large indels stand out. One of these was 17 bp long (between positions 43 and 59) in the ITS-1 region of the sequences 33 and 58 of *M. olivetorum*, and the other was 40 bp long (between positions 594 and 633) in the ITS-2 region of all *M. neglectum* sequences and the sequences 13 and 43 of *M. olivetorum*. The length of the remaining gaps varied between 1 bp and 3 bp.

Mean divergence value, or nucleotide diversity, for the ITS region of all species was 4.72%. The divergence values between *Botryanthus* species varied from zero (*M. cazorlanum*-1–*M. cazorlanum*-32, *M. neglectum*-22–*M. neglectum*-45, *M. baeticum*-18–*M. baeticum*-46) to 8.3% (*M. neglectum*-44–*M. olivetorum*-58). The divergence value increased to 10.1% when the outgroup species were considered.

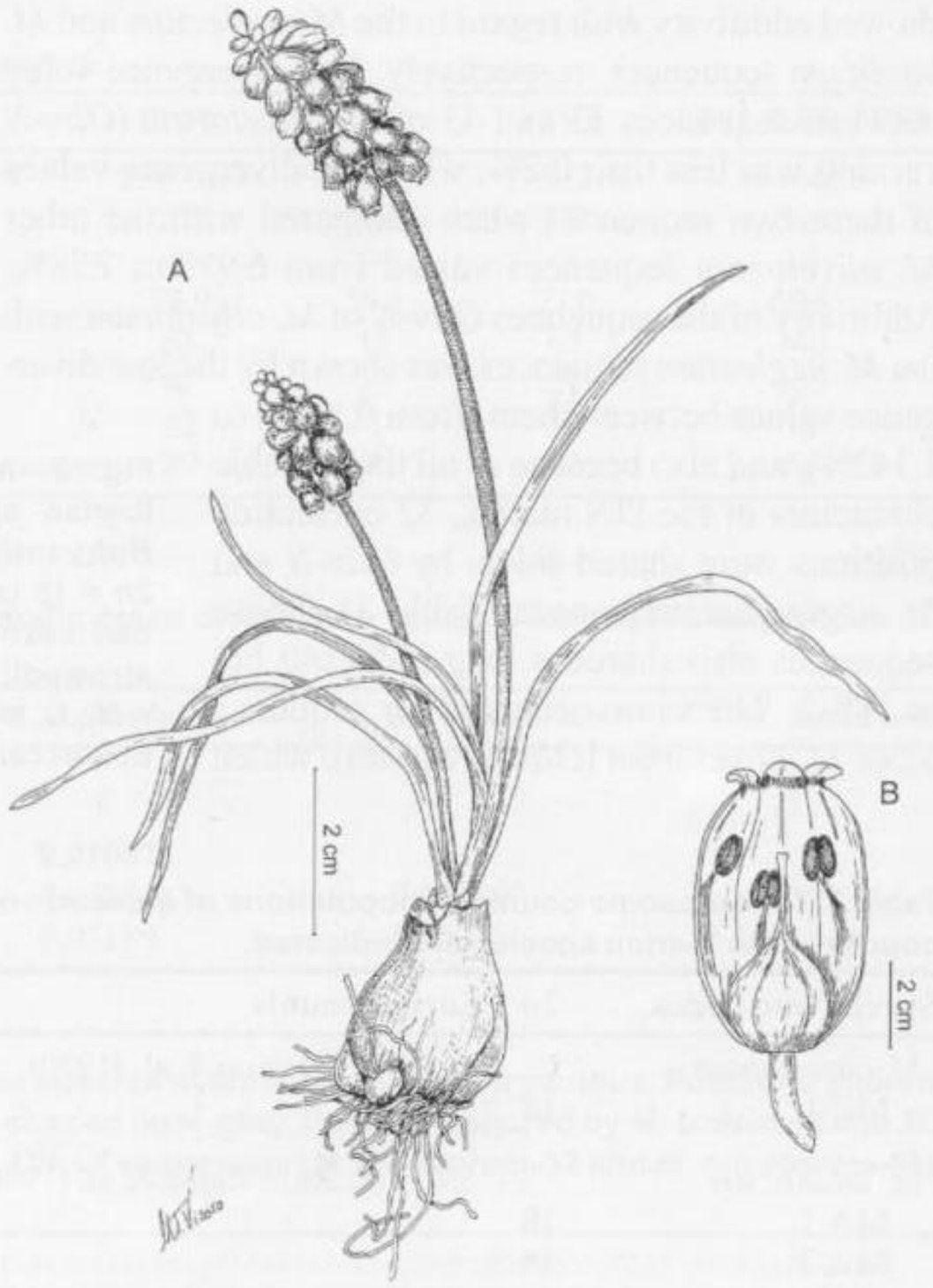

Fig. 3. *Muscari baeticum*. A, general appearance; B, fertile flower.

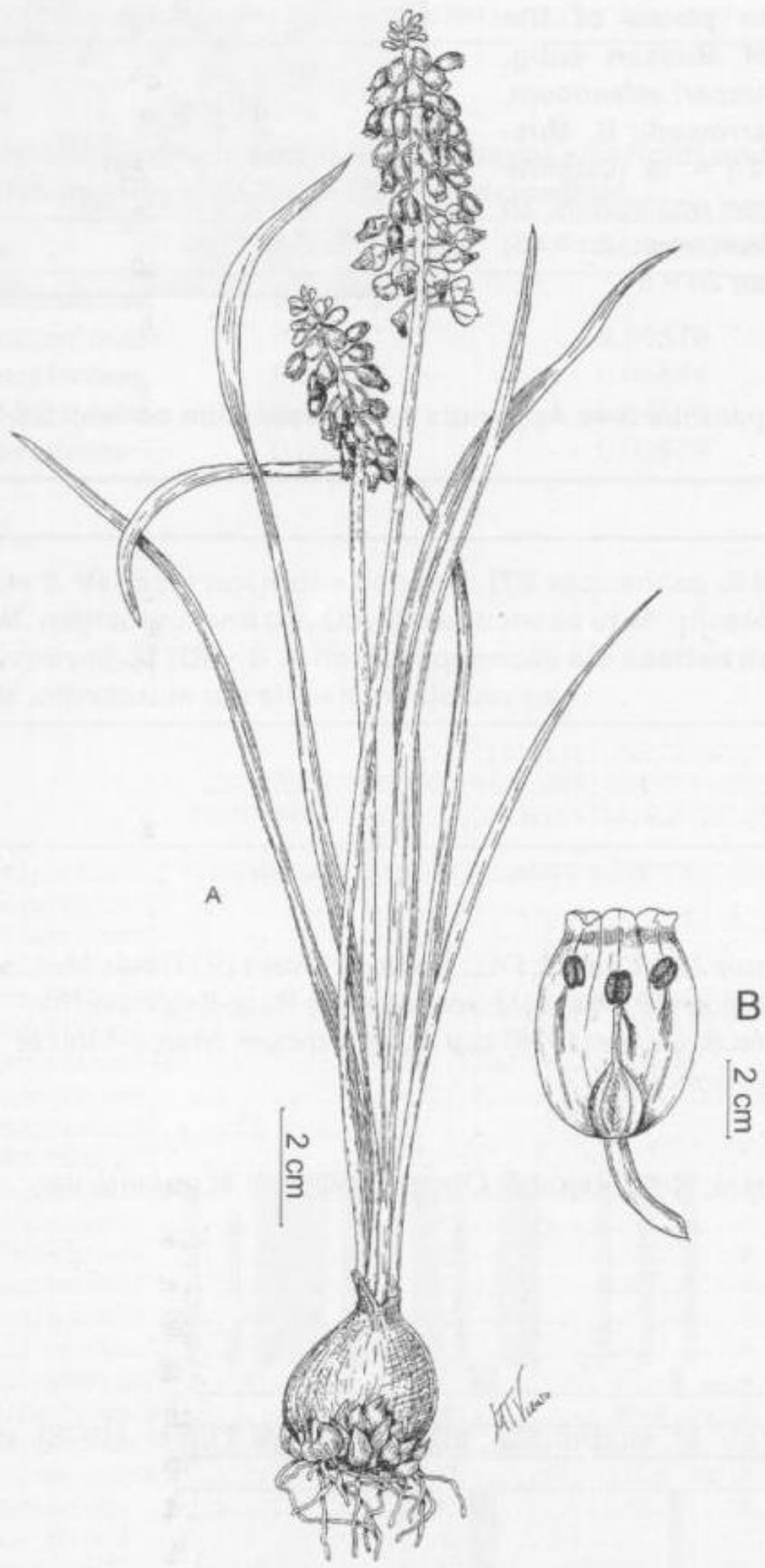

Fig. 2. *Muscari olivetorum*. A, general appearance; B, fertile flower.

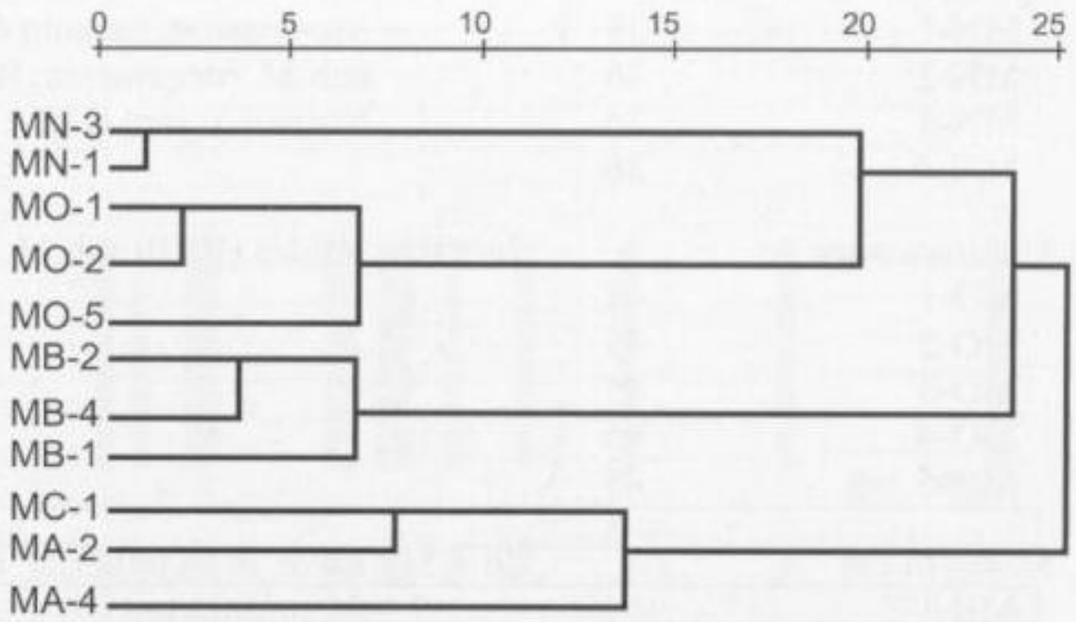

Fig. 4. UPGMA phenogram obtained from the euclidean distances calculated for the 19 morphological characters in 11 populations of *Muscari* subg. *Botryanthus* of the Iberian Peninsula. See Appendix 1 for population codes.

The high intraspecific divergence shown by the ITS sequences of *M. olivetorum* (higher than the interspecific values; Table 4) was due to the presence of different ITS variants in this species, which we named *Oliv-N* (sequences 13 and 43), *Oliv-B* (sequence 32) and *Oliv-X* (sequences 33 and 58). These ITS variants were demonstrated by the intraspecific divergence values and their nucleotide sequences. Moreover, the variants *Oliv-N* and *Oliv-B* showed additivity with regard to the *M. neglectum* and *M. baeticum* sequences, respectively. The divergence value between sequences 13 and 43 of *M. olivetorum* (*Oliv-N* variant) was less than 0.2%, while the divergence values of these two sequences when compared with the other *M. olivetorum* sequences varied from 6.9% to 7.78%. Additivity of the sequences *Oliv-N* of *M. olivetorum* with the *M. neglectum* sequences was shown by the low divergence values between them (from 0.168% to 1.342%), and also because of all the variable characters in the ITS matrix, 32 nucleotide positions were shared solely by *Oliv-N* and *M. neglectum* sequences (Table 5). These sequences also shared a long indel (40 bp) in ITS-2. The same occurred for sequence 32 of *M. olivetorum* (*Oliv-B* variant), which

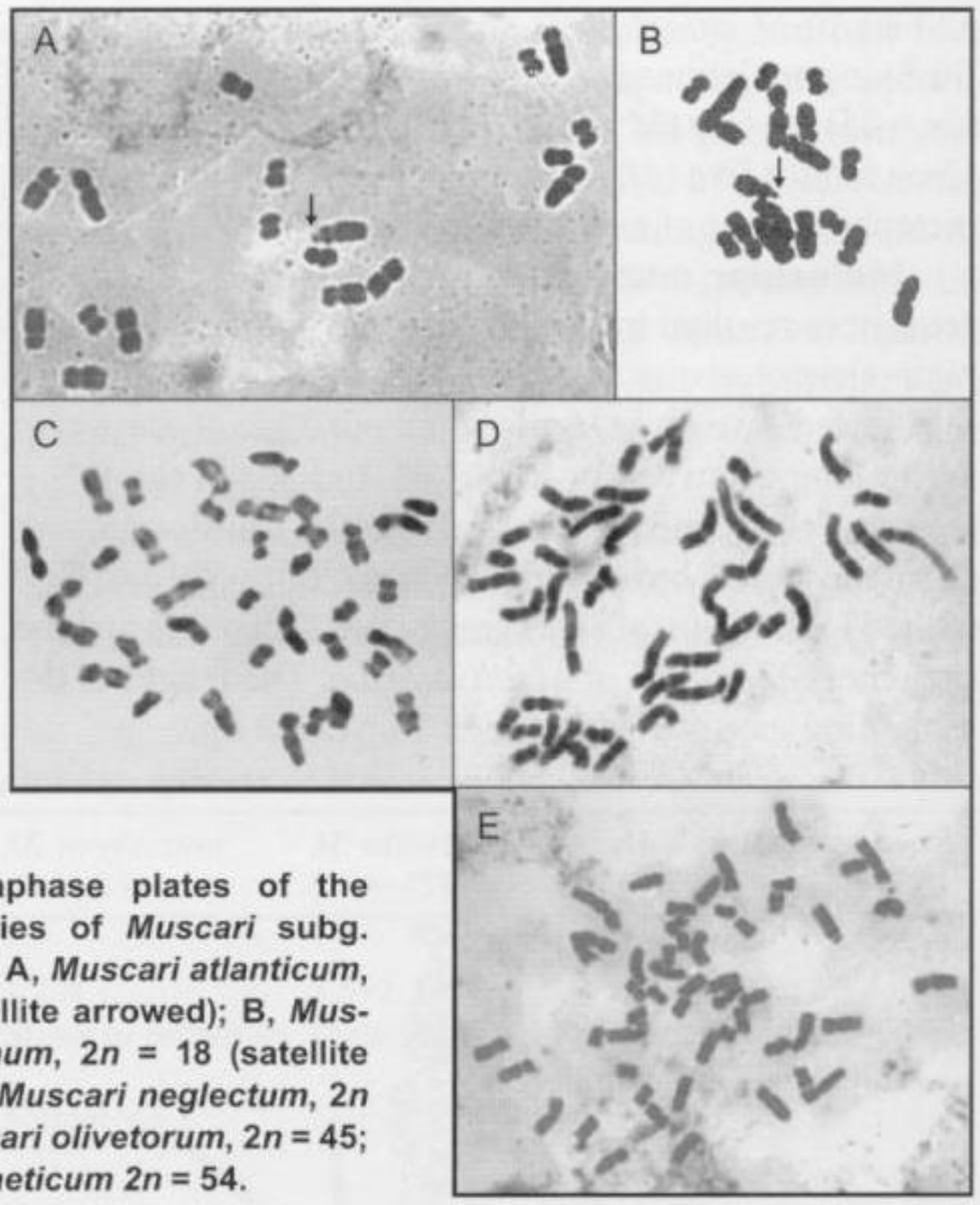

Fig. 5. Metaphase plates of the Iberian species of *Muscari* subg. *Botryanthus*. A, *Muscari atlanticum*, 2*n* = 18 (satellite arrowed); B, *Muscari cazorlanum*, 2*n* = 18 (satellite arrowed); C, *Muscari neglectum*, 2*n* = 36; D, *Muscari olivetorum*, 2*n* = 45; E, *Muscari baeticum* 2*n* = 54.

Table 2. Chromosome counts of populations of *Muscari* subg. *Botryanthus* (see Appendix 1 for population codes). Earlier counts of the Iberian species are indicated.

Species and codes	*2n*	Earlier counts
M. cazorlanum		*2n* = 18: Soriano & al. (1990)
MC-1	18	
M. atlanticum		*2n* = 18: Ruiz-Rejón & al. (1986)
MA-1	18	
MA-2	18	
MA-3	18	
MA-4	18	
MA-5	18	
MA-6	18	
M. neglectum		*2n* = 36: Valdés (1970) sub *M. racemosum* (L.) Lam. & DC.; Barros-Neves (1973) sub *M. racemosum*; Sañudo & Ruiz-Rejón (1975) sub *M. racemosum*; Ruiz-Rejón (1976) sub *M. racemosum*; Ruiz-Rejón & Oliver (1978) sub *M. atlanticum*; Marco-Moll & Notivol-Tejero (1979) sub *M. racemosum*
MN-1	36	
MN-2	36	
MN-3	36	
MN-4	36	
M. olivetorum		*2n* = 45: Valdés (1970) sub *M. racemosum*; Ruiz-Rejón & Oliver (1978) sub *M. atlanticum*
MO-1	45	
MO-2	45	
MO-3	45	
MO-4	45	
MO-5	45	
M. baeticum		*2n* = 54: Löve & Kjellqvist (1973) sub *M. atlanticum*; Ruiz-Rejón & Oliver (1978) sub *M. atlanticum*
MB-1	54	
MB-2	54	
MB-3	54	
MB-4	54	
MB-5	54	

showed high intraspecific divergence values (from 4.4% to 7.23%) and low divergence values with regard to the *M. baeticum* sequences (from 2% to 2.4%). Five of the nine apomorphies of *M. baeticum* sequences were present in *M. olivetorum*-32 (Table 5). Finally, sequences 33 and 58 of *M. olivetorum* (*Oliv-X* variant) showed low divergence values between each other (2.6%), and shared a 17 bp deletion in the ITS-1. However, they had high divergence values with regard to the other *M. olivetorum* sequences (from 4.40% to 7.78%). These two sequences showed the lowest interspecific divergence values with regard to the sequences of the diploid species (3.40%–4.87%). Table 5 shows the high nucleotide variability of the *Oliv-X* sequences.

Table 3. Features of the ITS sequences of species studied of *Muscari*.

	ITS1	5.8S	ITS2	ITS1-5.8S-ITS2
Length range (bp)	239–260	165	175–213	596–638
Aligned length (bp)	262	165	217	644
No. of gaps	8	–	7	15
G + C content mean (%)	72.9	56.1	77.0	69.8
No. of variable characters	63	15	62	140
No. of variable characters in *Muscari* subg. *Botryanthus*	41	13	51	105
No. of parsimoniously informative characters	42	4	30	76
No. of parsimoniously informative characters in *Muscari* subg. *Botryanthus*	29	3	26	58

Table 4. Intraspecific (principal diagonal and bold) and interspecific mean divergence values between ITS sequences of Iberian species of *Muscari* subg. *Botryanthus*.

	M. atlanticum	*M. cazorlanum*	*M. neglectum*	*M. olivetorum*	*M. baeticum*
M. atlanticum	**0.00632**				
M. cazorlanum	0.00602	**0.00570**			
M. neglectum	0.06972	0.06844	**0.01007**		
M. olivetorum	0.04956	0.04899	0.04802	**0.05609**	
M. baeticum	0.02883	0.02879	0.07189	0.04948	**0.00444**

Table 5. Variable positions between ITS sequences of the Iberian species of *Muscari* subg. *Botryanthus*. Positions shared by *M. neglectum* and the *Oliv-N* sequences of *M. olivetorum* are shaded light-grey. Positions shared by *M. baeticum* and *M. olivetorum*-32 (*Oliv-B* variant) sequences are shaded dark-grey. *Oliv-X* sequences (*M. olivetorum*-33 and *M. olivetorum*-58) of *M. olivetorum* are shown inside boxes.

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                    1111111111111222222222222233333333444444444444444444444455555555555555555555566666666666
                    1222335567788890012444556678902233334555570115568900223334445556677788899011112355555666678999000011233 44
                    9434516462292576190901237284318560278112342038047796707347356789670890364797347924234560279662672346040483 4
M.atlanticum5       CGCGATCCGCTCCTG-GGGCCTGACGGATGGTGGCGTCAGTATAGGCGTGGGCGGCGGCCCCAGGACGGCCGGCCCGAGCGAGGGAGAGTACCCGCCAGACCCACGG
M.atlanticum12      ..G.....-.....A.....A.....T.....C.....A.....
M.atlanticum15      .....-.....C.....C.....A.....A.....
M.atlanticum35      .....-.....C.....A..C.....
M.atlanticum39      .....-.....C.....C.....A.....
M.cazorlanum1       .....-.....C.....A.....
M.cazorlanum4       .....C.G.....A.....C.....A.A.....
M.cazorlanum6       .....-.....C.....A...A.....C.....A.....TT....
M.cazorlanum32      .....-.....C.....A.....
M.cazorlanum71      .....-.....T.....C.....A.....
M.neglectum10       T....GGTCTATG..CAACA..ATT....A.GT..A...A..A.....T....TG.....AT.AAC.T...A.C...T.A.....TT.A--------.TA
M.neglectum22       T..A.GGTCT.TG.ACAACA..ATT....A.GT..A...A..A.....A.A..TG.G....AT.AAC.T...A.C...T.A.....T..--------.TA
M.neglectum44       T..A.GGTCT.TG..CAACA..ATT....A.GC..A...A..A.....A.A..TG.G..G.AT.AAC.TT..A.CT..T.A.....T..--------.TA
M.neglectum45       T..A.GGTCT.TG.ACAACA..ATT....A.GT..A...A..A.....A.A..TG.G....AT.AAC.T...A.C...T.A.....T..--------.TA
M.neglectum70       T....GGTCT.TG..CAACA..ATT....A.GT..A...A..A.....C...A.A..TG.G....AT.AAC.T...A.C...T.A.....TT.A--------.TA
M.olivetorum13      T..A.GGTCT.TG..CAACA..ATT....A.GT..A...A..A...T.....A.A..TG.G....AT.AAC.T...A.C...T.A.....T..--------.TA
M.olivetorum32      ....T.....C..C.AC.....G...G..A....A...G.....G.T.....C...T...C.....A.....CAT..AGA..
M.olivetorum33      .A.....--.....C..C.....AG.A..G..A.....A.....G...T.....C....G.TC..A.GA...T..T..T..T.....A
M.olivetorum43      T..A.GGTCT.TG..CAACA..ATT....A.GT..A...A..A.....A.A..TG.G....AT.AAC.T...A.C...T.A.....T..--------.TA
M.olivetorum58      .....--.....C..C.....AT...AG.A..GT.A....T.A..A..A....A...G.....G..C.....C.A..GA...T....T..TT.....A
M.baeticum12        .....G..C.AC.....G..A...GA.....T....TGGT.....C.....C.....A.....A.C.T...G...
M.baeticum18        .....C..C.AC.....G..A...GA.....T.....GGT.....C.....C.....A.....C.T...G...
M.baeticum21        .....C..C.AC.....G..A...GA.....T.....GGT.G.....C.....C.....A....A.....C.T...G...
M.baeticum46        .....C..C.AC.....G..A...GA.....T.....GGT.....C.....C.....A.....C.T...G...
M.baeticum83        .....C..C.AC.....G..A...GA.G.....T.....GGT.....C.....C.....AG.....C.T...G...
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Parsimony analysis produced a most-parsimonious tree that was 158 steps long (CI: 0.93, RI: 0.966, HI: 0.07; CI: 0.879, HI: 0.121 without uninformative characters). The nucleotide substitution model selected by the likelihood ratio test for the ITS matrix was the transversional model (TVM). Maximum likelihood phylogenetic analysis produced one most-likely tree (–ln = 1801.316), which was exactly the same as MP tree (Fig. 6).

In the phylogenetic analysis (Fig. 6) the members of the subgenus *Botryanthus* form a strongly-supported monophyletic group. Within *Botryanthus* there are three principal clades. One includes all *M. neglectum* sequences together with the sequences of the *Oliv-N* variant of *M. olivetorum*. This clade is strongly supported by the bootstrap test, and is a sister clade to that formed by the other two principal clades, one of which is formed by the sequences of the two diploid species (*M. atlanticum* and *M. cazorlanum*) with the sequences of the *Oliv-X* variant of *M. olivetorum* (BS < 50%). The third major clade includes the sequences of *M. baeticum* (strongly grouped together) and the sequence of the *Oliv-B* variant of *M. olivetorum* (BS: 97/98). The relationship between the two major clades in which the diploids and the hexaploids appear has low bootstrap support.

DISCUSSION

Polyploidy is increasingly recognized as one of the major processes causing genetic diversity and speciation in plants, especially when matched to hybridization phenomena (Stebbins, 1950; Grant, 1971; Rieseberg, 1997; Wendel, 2000; Doyle & al., 2003). It has been argued that an ancestral polyploid event from a primitive Hyacinthaceae was responsible for the origin of the genus *Muscari*, (Garbari, 1972; Oliver & al., 1983). Moreover, within *Muscari* lineages, polyploidy is one of the principal processes that bring about species diversification (Bentzer, 1973; Karlén, 1984a, b). This is especially important in the subgenus *Botryanthus* (Garbari, 1974; Ruiz-Rejón & Oliver, 1978; Oliver & al., 1983; Karlén, 1984a, b), and within *Botryanthus* in the species complex called the *neglectum* group by Karlén (1984a), to which the species analyzed in this study belong.

Taxonomic characterization of the Iberian species of *Muscari* subg. *Botryanthus*. — Taxonomic characterization of the Iberian species of the *Muscari* subg. *Botryanthus* is based on their morphological and karyological characters, and on their ecological behaviour.

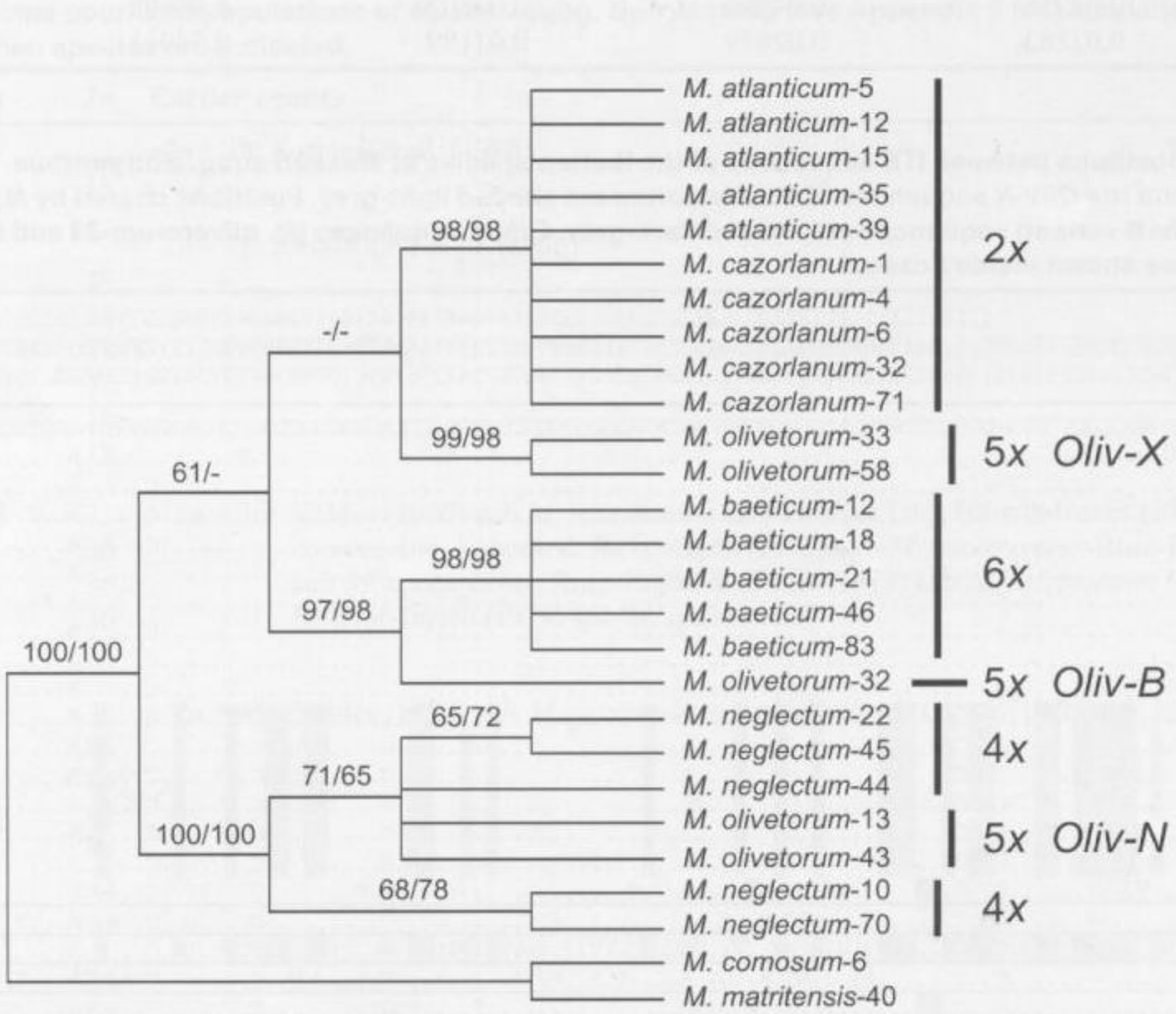

Fig. 6. Most-parsimonious tree, which coincides with the ML tree, obtained using the ITS sequences of *Muscari* species. Numbers above branches are the bootstrap values ≥ 50% (MP bootstrap/ML bootstrap). Ploidy levels of each species and the different ITS variants of *M. olivetorum* are indicated on the right.

Each species can be distinguished morphologically by a specific combination of characters. Thus, the diploid species differ from the polyploids by the location of their anthers, the quantity of sterile flowers, the length of the fertile-flower pedicels, and the length of the tepal lobes. However, the number of exclusive characters of the diploid species would be higher if it were not for the high number of characters shared between the diploids and *M. olivetorum* or *M. baeticum* (Table 1). When diploids share a character with *M. olivetorum*, they do not share it with *M. baeticum*, and vice versa. Frequently, this kind of distribution pattern of the morphological characters has been related to the probable hybrid origin of plant species.

The diploid species (*M. atlanticum*, *M. cazorlanum*) are morphologically quite distinct. The main differences are in the fertile-flower characters (colour, size and pedicel length), the colour of the bulb tunic and the length ratio between leaves and scape (Table 1). Both of them show a similar karyotype, but in *M. atlanticum* the satellite chromosome pair is the second pair, while in *M. cazorlanum* it is the sixth (Fig. 5). These results coincide with those obtained by Soriano & al. (1990). Despite all these differences, *M. atlanticum* and *M. cazorlanum* share many morphological characteristics (Table 1), which are clear signs of the close phylogenetic relationship between them. Garbari (2003) considered *M. cazorlanum* as a local variant of *M. atlanticum*. According to the results obtained in this study, we believe that *M. cazorlanum* and *M. atlanticum* are different species.

Many authors have pointed out the morphological differences between the different cytotypes of *M. neglectum*, but they consider that these phenotypic differences fall within the variability range of this species (cf. Garbari, 2003). However, our results show that morphologically speaking the three Iberian cytotypes (*M. neglectum*, *M. olivetorum*, *M. baeticum*) are clearly distinct from each other (Table 1). With regard to the identity of *M. neglectum*, as the type specimen of *M. neglectum* designated by Garbari (2003) shows the morphological characteristics of the tetraploids, the name *M. neglectum* corresponds to this cytotype. *Muscari olivetorum* differs from *M. neglectum* in that it is a more robust plant, and therefore has larger vegetative and floral structures, and long, lax inflorescences. *Muscari baeticum* differs from *M. neglectum* in the bulb and fertile-flower characteristics, scape diameter, and leaf direction. Differences between the pentaploid and hexaploid cytotypes and the tetraploid have also been identified using isoenzymes. Oliver & Ruiz-Rejón (1980) analyzed esterases in 1,017 individuals from 30 Iberian populations of *M. neglectum* s.l., and found a relationship between the chromosomal level and the isoenzyme pattern. Furthermore, in another isoenzymatic analysis Oliver & al. (1983) identified the existence of two species within *M. racemosum* (L.) Lam. & DC. (syn. *M. neglectum*), which they called A group and B group. The A group included all tetraploid individuals and several pentaploids, while the B group contained several pentaploid individuals and all the hexaploids. These results support the presence within *M. olivetorum* of intermediate morphological characters that fall between those of *M. neglectum* and *M. baeticum* (length of tepal-lobes, diameter of the perianth mouth, stipule colour) and characters shared with one or both of them (bulb-tunic colour, quantity of bulbils, leaf direction, fertile-flower colour, anther location, quantity of sterile-flowers). This indicates a possible hybrid origin of *M. olivetorum*.

UPGMA phenogram results support the taxonomic recognition and characterization of *M. olivetorum* and *M. baeticum* (Fig. 4). The phenogram shows that the populations group together by taxonomic affinity, indicating the interpopulational homogeneity of the morphological characters of each species.

The two diploid species, *M. atlanticum* and *M. cazorlanum*, inhabit low woody scrublands or ledges on calcareous rock formations, at low to medium altitude (Table 1). The distribution area of *M. cazorlanum* is restricted to one population in the Sierra de Cazorla mountains (province of Jaén), while *M. atlanticum* is found in the S and SE coast of the Iberian Peninsula. *Muscari neglectum* and *M. olivetorum* always inhabit cultivated lands with strong human influence. *Muscari baeticum*, however, occurs in low woody scrublands or ledges of calcareous rock formations, and thrives at altitudes of over 1,300 m, although it also grows in cold, humid places at lower elevations. *Muscari neglectum* inhabits low to medium altitudes (Table 1). *Muscari olivetorum* occupies an area between *M. neglectum* and *M. baeticum* that overlaps with both these species.

Phylogenetic analysis and evolutionary pattern of the subgenus *Botryanthus* in the Iberian Peninsula. — Phylogenetic analysis of the ITS region of the five species of the subgenus *Botryanthus* found in the Iberian Peninsula has enabled us to establish the relationships between them and to establish the most probable evolutionary pattern.

Phylogenetic analysis confirms the close relationship between the two diploid species, *Muscari atlanticum* and *M. cazorlanum*. Within the strongly supported clade, there is no separation of sequences, which form a large polytomy (Fig. 6). This is reflected in the divergence matrix (Table 4), where intraspecific values are similar to the divergence value between them. At the sequence level, the only difference between the species is a gap (position 577 of the alignment) in the *M. cazorlanum* sequences. This indicates a short divergence time from the speciation event that separated *M. atlanticum* and *M. cazorlanum*.

The disjointed distribution pattern of *Muscari atlanticum*, which is restricted to isolated populations in

the warm places of the S and SE coast of the Iberian Peninsula and Morocco, suggests that it is an old species (the last separation between the south of the Iberian Peninsula and Morocco was at the end of the Pliocene) whose populations were reduced and confined to warm places during the glaciations of the Quaternary. According to this theory, and with the most probable evolutionary direction (from a parsimonious point of view) of the morphological characters in the ITS phylogeny, we hypothesize that *M. cazorlanum* could have diverged from *M. atlanticum* by an allopatric speciation process caused by genetic isolation and genetic drift of the *M. cazorlanum* population. Allopatric speciation models have been described for many plants in the Iberian Peninsula (Beerli & al., 1996; Caujapé-Castells & Jansen, 2003; Coleman & al., 2003; Navarro & al., 2004).

With regard to the polyploid species, *Muscari baeticum* and *M. olivetorum* have been considered autopolyploid cytotypes of *M. neglectum* (Ruiz-Rejón & Oliver, 1978; Oliver & Ruiz-Rejón, 1980; Oliver & al., 1983). Our results, however, do not support this hypothesis. The high differentiation of the ITS sequences of *M. baeticum* from those of *M. neglectum* (Table 4), and the molecular and morphological relationships with *M. atlanticum* (Fig. 6, Tables 1, 4), support an autopolyploid origin of *M. baeticum* from an ancestor related to *M. atlanticum* (and not to *M. neglectum*). Moreover, the high divergence between the ITS of *M. neglectum* and *M. atlanticum*, combined with the presence of a diploid cytotype of *M. neglectum* in the eastern Mediterranean (Greece and Turkey), indicate that tetraploid *M. neglectum* is not from the same lineage as the diploids of the Iberian Peninsula and nor, therefore, from the same lineage as *M. baeticum*.

A number of hypotheses can be proposed for the autopolyploid origin of *M. baeticum*, in which the mediation of an intermediate-stage polyploid that originated from an ancestral diploid is always necessary. However, participation of *M. neglectum* in the origin of *M. baeticum* cannot be excluded if we consider the morphological characters shared by the two species (Table 1). Thus, the presence in *M. baeticum* of morphological characters shared with both *M. neglectum* and *M. atlanticum* supports the allopolyploid origin of *M. baeticum* from both these species (or their ancestors). Given the uniformity of the ITS sequences of *M. baeticum* and their high differentiation from *M. neglectum*, however, an allopolyploid origin of *M. baeticum* would imply the biased homogenization of its paralogous ITS toward the ITS of the diploid donor. In allopolyploids, biased homogenization of the nrDNA toward one of the parental types has been reported in many studies (Wendel & al., 1995; Brochmann & al., 1996; Roelofs & al., 1997; Ferguson & al., 1999; Franzke & Mummenhoff, 1999; Fuertes-Aguilar & al., 1999a, b; Wendel & Cronn, 2003).

As for *Muscari olivetorum*, its different ITS variants and their position in the phylogenetic tree contradict the hypothesis of an autopolyploid origin of this species from *M. neglectum* but support a hybrid origin. ITS additivity in *M. olivetorum* with respect to the sequences of *M. neglectum* and *M. baeticum* (*Oliv-N* and *Oliv-B* variants, respectively) supports the idea that these species are the parents of *M. olivetorum*. This is coincident with the results obtained by Oliver & al. (1983) using isoenzymatic markers.

Additivity of the *Oliv-N* and *Oliv-B* sequences in *M. olivetorum* indicates that the homeologous nrDNA repeats have been retained in the allopolyploid, hence the absence of interlocus homogenization between homeologous loci. This phenomenon has been observed in several allopolyploid plants (cf. Álvarez & Wendel, 2003), such as *Tragopogon* (Soltis & Soltis, 1991; Soltis & al., 1995) and *Spartina* (Baumel & al., 2001).

The absence of interlocus homogenization of the nrDNA homeologues in *M. olivetorum* could be explained, in addition to its recent origin, by the absence of sexual reproduction. Ruiz-Rejón & Oliver (1978) detected meiotic aberrations in *M. olivetorum* (premature segregation of univalents, lagging chromosomes in anaphase-I and anaphase-II, chromosome bridges, and micronucleus in telophase-I and telophase-II) with inviable gametes. Any viable gametes would be unbalanced. New pentaploid individuals therefore would only be produced in the unlikely event that complementary gametes were to fuse. This asexuality will prevent establishment of homogenized variants. Thus, nrDNA homeologues will survive in the allopolyploid, as has been shown in *Paeonia* (Sang & al., 1995; Li & Zhang, 2002) and *Amelanchier* (Campbell & al., 1997).

NrDNA expression in allopolyploids seems to be influenced by the phenomenon of nucleolar dominance (Reeder, 1985; Chen & Pikaard, 1997; Pikaard, 2000), which can affect interlocus homogenization of the nrDNA homeologues. If one of the homeologous ribosomal loci is inactivated by a high degree of methylation, it will remain condensed during interphase, so homeologues cannot contact each other in the nucleolus preventing interlocus homogenization. This has been shown to occur in allopolyploids of *Nicotiana* (Lim & al., 2000). In *M. olivetorum*, evidence from both nucleotide divergence values and the characteristics of the sequences of the *Oliv-X* variant suggest the possible influence of epigenetic modifications in the absence of interlocus homogenization between the homeologous nrDNA loci.

In *Muscari olivetorum*, nucleotide divergence values are very low between the sequences of the *Oliv-N* variant and those of *M. neglectum* (0.168%–1.342%), while they are higher between the sequence of the *Oliv-B* variant and the sequences of *M. baeticum* (2.054%–2.392%). Therefore, the sequence of the *Oliv-B* variant has undergone more changes than the sequences of the *Oliv-N* variant since the origin of *M. olivetorum*. Different evolutionary

rates between nrDNA sequences belonging to the same genome have been used as evidence of transcriptional inactivity of those sequences with higher rates, due to relaxed functional pressure (cf. Bailey & al., 2003). This would suggest possible inactivation of the ribosomal loci donated by *M. baeticum* in *M. olivetorum*. The sequences of the *Oliv-X* variant of *M. olivetorum* support this hypothesis. They share a 17 bp deletion in ITS-1, and show high divergence values with respect to *M. baeticum* and the *Oliv-B* variant of *M. olivetorum*. However, phylogenetic analysis shows that these sequences are related to those of the diploids and of *M. baeticum*. The characteristics of these sequences perhaps indicate that they are inactive sequences (free of functional pressure) that remain condensed during interphase. This supports the hypothesis of epigenetic modifications of the ribosomal loci donated by *M. baeticum* preventing interlocus homogenization of nrDNA homeologues in *M. olivetorum*.

According to Stebbins (1950), polyploidy should be more frequent in plants with vegetative or apomictic reproduction systems than in those lacking of these systems, because they can produce more individuals of the same cytotype to breed with, or do not even require cross-reproduction. As far as *M. olivetorum* is concerned, its considerable ability for vegetative reproduction, producing up to 50 bulbils per bulb, may have facilitated the establishment of populations.

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Appendix 1. Species and populations of *Muscari* studied in the Iberian Peninsula.

Species: country: population code (in bold); locality; altitude (m a.s.l.); voucher; type of analysis; EMBL accession (in parenthesis).

***M. atlanticum*:** Spain: **MA-1**; Cádiz, Olvera, peñón de Zaframagón; 500; *120993* (SEV); M, C. **MA-2**; Granada, Vélez de Benaudalla, la Bernardilla; 100; *M. Ruiz Rejón, 19/III/1994, 37766* (GDAC); M, C, I (AM116981, AM116982, AM116983, AM116984, AM116985). **MA-3**; Jaén, road Jaén–Granada, Cambil-Huelma crossing; 600; *G. Blanca, 7/IV/1997, 41984* (GDAC); M, C. **MA-4**; Málaga, Casarabonela, Sierra Prieta; 900; *G. Blanca & M. Ruiz Rejón, 16/V/1998, 44693* (GDA); M, C. **MA-5**; Sevilla, Peñón de Algámitas; 500; *120992* (SEV); M, C. **MA-6**; Valencia, Beniatjar, Sierra de Benicadell; 870; *G. Blanca & M.J. Salinas, 13/V/1997, 41985* (GDAC); M, C. ***M. cazorlanum*:** Spain: **MC-1**; Jaén, Hornos, pantano del Tranco; 700; *A. Benavente, 6/V/1998, 44694* (GDA); M, C, I (AM116986, AM116987, AM116988, AM116989, AM116990). ***M. neglectum*:** Spain: **MN-1**; Granada, Albolote, Cortijo del Aire; 750; *C. Morales, 22/III/1988, 31119* (GDAC). **MN-2**; Granada, Llano de la Perdiz; 980; *P.A. Burgos, 9/IV/1988, 32763* (GDAC); M, C. **MN-3**; Granada, Padul; 800; *M. Ruiz Rejón, 21/VI/1979, 14159* (GDAC); M, C, I (AM116991, AM116992, AM116993, AM116994, AM116995). **MN-4**; Granada, Sierra Elvira; 750; *M.T. Vizoso Paz & A. Vizoso González, 23/III/1989, 43748* (GDAC); M, C. ***M. olivetorum*:** Spain: **MO-1**; Granada, Guadahortuna; 970; *M. Ruiz Rejón, 8/III/1998, 42392* (GDAC); M, C. **MO-2**; Jaén, road Peal de Becerro–Hornos de Peal; 500; *M. Ruiz Rejón, 8/III/1998, 42391* (GDAC); M, C. **MO-3**; Jaén, Torredelcampo, Cerro Morteros; 1,000; *G. Blanca, 11/IV/2001, 44692* (GDA); M, C, I (AM260545, AM260546, AM260547, AM260548, AM260549). **MO-4**; Jaén, Sierra de Alta Coloma; 900; *P. Navarro, 28/XI/1990, 35396* (GDAC); M, C. **MO-5**; Jaén, Sierra de Cazorla, between La Iruela and Burunchel; 800; *M. Ruiz Rejón, 8/III/1998, 42393* (GDAC); M, C. ***M. baeticum*:** Spain: **MB-1**; Granada, Sierra de Cázulas; 1330; *M. Ruiz Rejón, 3/IV/1994, 37848* (GDAC); M, C, I (AM260550, AM260551, AM260552, AM260553, AM260554). **MB-2**; Granada, Sierra Nevada, Dornajo; 1900; *M. Ruiz Rejón, 23/V/1997, 41983* (GDAC); M, C. **MB-3**; Granada, Sierra Nevada, Trevenque; 1,700; *G. Blanca & M. Ruiz Rejón, VI/1990, 44689* (GDA); M, C. **MB-4**; Jaén, Sierra de Cazorla, embalse del Tranco; 750; *G. Blanca, A. Benavente & M. Ruiz Rejón, 7/IV/1998, 44691* (GDA); M, C. **MB-5**; Jaén, Sierra de Mágina, Cárceles; 1,900; *G. Blanca & M. Ruiz Rejón, 20/VI/1991*, 44687 (GDA); M, C. ***M. comosum*:** Spain: **COM**; Granada, Padul; 800; *M. Ruiz Rejón, III/1974, 14255* (GDAC); I (AM117020). ***M. matritensis*:** Spain: **MAT**; Granada: Sierra Nevada, Dornajo; 1,900; *M. Ruiz Rejón, 21/VI/1998, 42800* (GDAC); I (AM117025).

GDA, GDAC: herbarium of the University of Granada; SEV: herbarium of the University of Sevilla; M: morphological analysis; C: cytogenetical analysis; I: molecular analysis.

Appendix 2. Key to species of *Muscari* subg. *Botryanthus* in the Iberian Peninsula.

1. Fertile flowers greenish ..... *M. cazorlanum*
1. Fertile flowers blue 2
2. Bulbils numerous; fertile flowers dark-blue 3
2. Bulbils 0–1(–2); fertile flowers bluish-violet or bluish-purple 4
3. Inflorescence short and dense; scapes 1.5–2.0 mm in diameter; leaves 1.8–2.5 mm wide; pedicels 2–3 mm; anthers 0.8–1.0 mm ..... *M. neglectum*
3. Inflorescence long and lax; scapes (2.0–)2.5–3.5 mm in diameter; leaves 2.5–6.0 mm wide; pedicels 3.5–4.0 mm; anthers 1.0–1.2 mm. ..... *M. olivetorum*
4. Inflorescence long and lax; bulb (1.5–)1.8–2.4 cm wide; anthers 1.0–1.2 mm, exserted; leaves as long as or shorter than scapes in flowering ..... *M. atlanticum*
4. Inflorescence short and dense; bulb 1.2–1.6 cm wide; anthers 1.3–1.5 mm, included; leaves 1.0–1.5(–2.0) as long as scapes in flowering ..... *M. baeticum*

Appendix 3. Diagnosis and description of the two new species.

Muscari olivetorum Blanca, M. Ruiz-Rejón & V.N. Suárez-Santiago, **sp. nov.** (Fig. 2).

Validior quam *M. neglectum*, a quo differt scapis (2.0–)2.5–3.5 mm diam., foliis (2.5–)3.0–6.0 mm latis, inflorescentia longiore laxaque, pedicellis 3.5–4.0 mm longis, antheris 1.0–1.2 mm item longis chromosomaticoque numero $2n = 45$.

Holotypus. Torredelcampo (in Hispaniae provincia giennensi), 700 m, in oliveto, ubi G. Blanca legit mense, Aprili anni 1974 (GDAC 342).

Description. Perennial herbaceous, scapous, bulbous plant. Bulb 1.5–2.5 cm in diameter, ovoid, with membranous outer tunics with a light straw colour; bulbils numerous (20–50). Scapes 1–2(–3) per bulb, simple, 130–200 × (2.0–)2.5–3.5 mm. Leaves 1–2 as long as scape, (2.5–)3.0–6.0 mm wide, basal, arched-ascending, sessile, simple, subcylindric, canaliculate, ± glaucous, parallel-nerved, with 13–14 nerves. Inflorescence racemose simple, with long and lax raceme, bracteate; bracts ovate or linear, bluish-white; pedicels 3.5–4.0 mm in anthesis, shorter than perianth. Flowers actinomorphic, hermaphroditic, trimerous (apparently hexamerous); apical flowers esterile, less than 20% in total, pale-blue. Lower flowers fertile, with perianth 5.5–6.5 × 3.5–4.2 mm, urceolate, constricted in the apex, dark-blue; tepals joined almost all the way down, with six apical lobes; lobes 1.0–1.2 mm, recurved, broadly ovate, obtuse, white or bluish-white towards the base; perianth mouth c. 2.2 mm in diameter. Androecium with 6 stamens, 3 inserted in the middle of the perianth and another 3 in the upper third. Anthers 1.0–1.2 mm, attached by middle, introrse, included. Ovary superior, 3-chambered, with 1 style included; stigma broadened and faintly three-lobed. Capsule membranous, strongly three-lobed, emarginate or truncate; seeds ellipsoidal or subglobose, black.

Muscari baeticum Blanca, M. Ruiz-Rejón & V.N. Suárez-Santiago, **sp. nov.** (Fig. 3).

Differt a M. neglecto bulbulis paucioribus (0–2), floribus fertilibus (6.8–)7.0–8.2 × 3.5–5.0 mm longis latisque, caeruleo-violaceis vel caeruleo-purpureis, antheris 1.3–1.5 mm longis chromosomaticoque numero $2n = 54$; differt autem a M. atlantico bulbo minore (1.2–1.6 cm lato), foliis florescendi tempore 1.0–1.5(–2.0)-plo longioribus quam scapis, inflorescentia brevi densaque, antheris 1.3–1.5 mm longis, valde inclusis, et chromosomatico numero $2n = 54$.

Holotypus. Sierra Nevada, Dornajo (in Hispaniae provincia granatensi), 1950 m, in genistetis, locis petrosis, dolomiticis, ubi M. Ruiz Rejón legit die 30 mensis Iunii anni 1998 (GDA 44686).

Description. Perennial herbaceous, scapous, bulbous plant. Bulb 1.2–1.6 cm in diameter, ovoid, with dark brown membranous outer tunics; bulbils absent or scant (0–2). Scapes 1–2(–3) per bulb, simple, 110–170 × 2.0–2.8 mm. Leaves 1.0–1.5(–2.0) as long as scape and 2.0–2.8 mm wide, basal, prostrate, sessile, simple, subcylindric, canaliculate, ± glaucous, parallel-nerved, with 11–12 nerves. Inflorescence racemose simple, with short and dense raceme, bracteate; bracts ovate or linear, white; pedicels 3.0–3.5 mm in anthesis, shorter than perianth. Flowers actinomorphic, hermaphroditic, trimerous (apparently hexamerous); apical flowers sterile, less than 20% in total, blue. Lower flowers fertile, with perianth (6.8–)7.0–8.2 × 3.5–5.0 mm, urceolate, constricted at the apex, bluish-violet or bluish-purple; tepals joined almost all the way down, with six apical lobes; lobes 1.0–1.5 mm, recurved, broadly ovate, obtuse, white; perianth mouth 1.6–3.0 mm in diameter. Androecium with 6 stamens, 3 inserted in the middle of the perianth and another 3 in the upper third. Anthers 1.3–1.5 mm, attached by middle, introrse, included. Ovary superior, 3-chambered, with 1 style included; stigma broadened and faintly three-lobed. Capsule membranous, strongly three-lobed, emarginate or truncate; seeds ellipsoidal or subglobose, black.