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Nucleolar number variation in *Fritillaria* (Liliaceae) taxa from Greece

Sofia Samaropoulou[1*], Pepy Bareka[1], Aggelos Kanas[1] and Georgia Kamari[2]

1 Laboratory of Systematic Botany, Faculty of Crop Science, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece
2 Laboratory of Botany, Section of Plant Biology, Department of Biology, University of Patras, GR26500 Patras, Greece
* Correspondence: s.samarop@gmail.com

ABSTRACT:
The current study aims to estimate the most frequent number of nucleoli in taxonomically interesting *Fritillaria* species. Silver nitrate staining is applied using a modified protocol in 14 taxa and one hybrid from 17 populations from Greece. This is the first report of the number of nucleoli for all the *Fritillaria* taxa studied here. In general, the number of nucleoli ranges from 0-8, with *Fritillaria pontica* and *F. theophrasti* characterised by the greatest number of observed nucleoli. The results reinforce the classification of taxonomically intriguing taxa, such as *Fritillaria sporadum* and *F. theophrasti,* as distinct species, even though they have recently been considered synonyms of *F. ehrhartii* and *F. pontica*, respectively.

Keywords:
karyology, nucleoli, $AgNO_3$, NORs, Greek endemics

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INTRODUCTION

The karyology of the genus *Fritillaria* L. has been the subject of study for many years due to its large genome size (Darlington 1935, 1937; Frankel 1940). *Fritillaria* taxa are characterised by quite a stable karyotype morphology and their karyotypes are usually diploid and asymmetrical, with a basic chromosome number of $x = 12$. Only a few species are an exception to this pattern, with a basic chromosome number of $x = 9$ (three species), $x = 11$ (two species) and $x = 13$ (two species). However, basic chromosome numbers other than $x = 12$ are considered to have derived from fusion or fission (La Cour 1978a; Kamari 1991a). Moreover, a few cases of triploidy have also been mentioned, i.e. $2n = 3x = 36$ chromosomes (Fedorov 1969; La Cour 1978a; Marchant & Macfarlane 1980; Moore 1982; Peruzzi *et al.* 2009; Zaharof 1987, unpublished) or $2n = 3x = 27$ chromosomes (Cesca 1986; Kamari 1991a; Samaropoulou *et al.* 2016).

On the other hand, the presence and morphology of satellited chromosomes vary among the karyotypes of *Fritillaria* taxa or even among populations of the same species (Runemark 1970; Bentzer *et al.* 1971; Mehra & Sachdeva 1976; Koul & Wafai 1980; Kamari 1984a, 1991a, 1996; Kamari & Phitos 2006; Samaropoulou *et al.* 2016, 2019a). In addition, secondary constrictions (SC), as well as B-chromosomes are often observed (La Cour 1978b, c; Kamari 1984a, 1991a, b; Zaharof 1989; Kamari & Phitos 2006). Consequently, particular focus is placed on such differentiations in order to detect the differences among the generally stable karyotypes of *Fritillaria* taxa, and specific chromosome pairs are studied as markers (Kamari 1984b; Zaharof 1989; Kamari & Phitos 2000, 2006).

Karyotypes of *Fritillaria* taxa from Greece have a basic chromosome number of $x = 12$, apart from *F. montana* Hoppe ex W.D.J. Koch, whose basic chromosome number is $x = 9$. Classic karyological study has been applied on most of the taxa, however, due to the aforementioned stable karyotype morphology, the results are not always able to interpret the taxonomic relations among the taxa.

Silver nitrate staining is one of the most common and efficient techniques to detect nucleoli and active nucleolar organiser regions (NORs), which are usually located

Table 1. Populations of *Fritillaria* taxa studied with their voucher numbers, number of secondary constrictions (SC), range and most frequent number of silver-stained nucleoli.

Taxon	Population	Voucher no.	No. of SC	Range of nucleoli no.	Most frequent nucleoli no.
F. ehrhartii Boiss. & Orph.	Aegean Isl., Cyclades Isl., Tinos	SF1109	8	1-3	2
F. elwesii Boiss.	Aegean Isl., Kastellorizo, Agios Georgios	171521	0	2-7	4-5
F. graeca Boiss. & Spruner	Sterea Ellas, Attika, Isl. Salamina	SF1051	4	2-3	2
F. messanensis subsp. *messanensis* Rafin.	Macedonia, Pieria, Mt. Olympus	SF1084	7	1-5	3
	Peloponnisos, Ilia, Iraklia	F4	4	1-4	3
F. messanensis subsp. *gracilis* (Ebel) Rix	Ionian Isl., Kefalonia	SF1053	4	1-2	1
F. messanensis subsp. *sphaciotica* (Gand.) Kamari & Phitos	Aegean Isl., Kriti, Mt. Psiloritis	F32	6	1-4	2
F. mutabilis Kamari	Ionian Isl., Kefalonia	SF1070	6	1-3	2-3
F. obliqua subsp. *obliqua* Ker-Gawler	Sterea Ellas, Attika, Marathonas	SF1052	4	3-5	3
F. obliqua subsp. *tuntasia* (Heldr. ex Halácsy) Kamari	Aegean Isl., Serifos	SF1064	4	1-4	3
F. pontica Wahlenb.	Thraki, Komotini	SF1077	2	3-8	5
F. rhodocanakis Orph.	Sterea Ellas, Attika, Isl., Idra	SF1104	6	1-4	4
F. spetsiotica Kamari	Peloponnisos, Argolida, Nafplio	SF1098	6	2-5	2-5
F. spetsiotica × *F. rhodocanakis* ($2n = 2x$)	Peloponnisos, Argolida, Epidavros	SF1099	4	1-5	2-3
F. spetsiotica × *F. rhodocanakis* ($2n = 3x$)	Peloponnisos, Argolida, Didima	SF1100	3	1-5	2-3
F. sporadum Kamari	North Sporades Isl., Gioura	SF1107	4	1-6	2-3
F. theophrasti Kamari & Phitos	East Aegean Isl., Lesbos	SF1071	2	2-5	2-4

on secondary constrictions (Ferguson-Smith 2013), and their determination can provide useful information and make a significant contribution to evolutionary and cytogenetic studies (Galetti 1998). For the above reasons, in the present study, the number of nucleoli has been estimated in representatives of the genus *Fritillaria* from Greece, with particular attention to endemic species, as well as to taxa of taxonomic interest, as part of a wider karyological and biosystematic study of this taxonomically intriguing genus (Kamari 1984a, b, 1991a, b, 1996; Kamari & Phitos 2000, 2006; Samaropoulou *et al.* 2016, 2019a, b, 2021).

MATERIALS AND METHODS

The number of nucleoli was determined in 14 *Fritillaria* taxa and one hybrid with different levels of ploidy in material from Greece (Table 1) using the protocol proposed by Kavalco & Pazza (2004) with some modifications. Pretreated root tips (Samaropoulou *et al.* 2019a) were hydrolysed in 1N HCl at 60°C for 8 min and immersed in warm water. The material was then placed on a slide with two drops of aqueous gelatin solution (1%) and four drops of aqueous $AgNO_3$ solution (25% with two drops of formic acid). After being gently squashed, the stained root tips were heated at 60°C for 30 min.

At least one hundred cells per taxon were observed using an AXIOLAB Zeiss microscope and photographed with a Canon EOS 600D digital camera (Fig. 1). The proportional frequency of the nucleolar number per taxon was demonstrated in the form of histograms using Microsoft Office Excel (Figs. 2–4).

RESULTS AND DISCUSSION

Fritillaria representatives from 17 populations from Greece were studied and, to our knowledge, this is the first time that silver nitrate staining has been applied, and the number of nucleoli determined in all these studied *Fritillaria* taxa. The results are summarised in Table 1 and indicatively presented in Fig. 1. The number of nucleoli, and consequently the evaluation of the active nucleolar organiser regions (NORs), were determined by observing interphase nuclei.

Fritillaria ehrhartii Boiss. & Orph. and *F. sporadum* Kamari have recently been considered as synonyms (Dimopoulos *et al.* 2013; Strid 2016). However, in addition to differences in the main morphological features (Kamari 1984a, b), karyotype formula (Kamari 1984a, b; Samaropoulou 2021, unpublished), as well as seed and pollen morphology (Samaropoulou *et al.* 2019b, 2021), the range in the number of nucleoli also differs. More precisely, the number of nucleoli ranges from 1 to 6 per nucleus in the case of *F. sporadum* (Fig. 4C), while the maximum number of observed nucleoli was three in *F. ehrhartii* (Fig. 2A). Previous karyological studies of

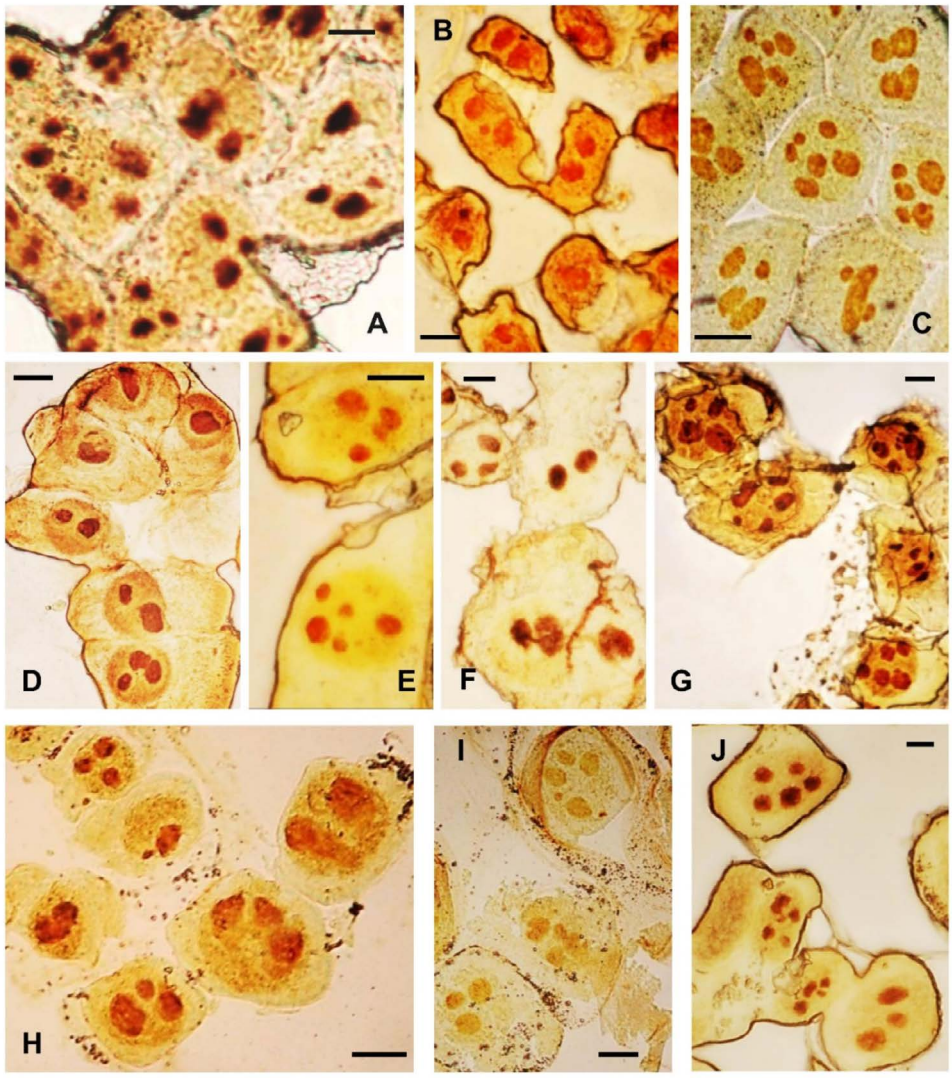

Fig. 1. Photomicrographs of interphase nuclei after silver nitrate staining. **A)** *F. drenovskii*; **B)** *F. graeca*; **C)** *F. elwesii*; **D)** *F. messanensis* subsp. *messanensis* (SF1084); **E)** *F. obliqua* subsp. *obliqua*; **F)** *F. obliqua* subsp. *tuntasia*; **G)** *F. pontica*; **H)** *F. spetsiotica* × *F. rhodocanakis* ($2n = 2x$); **I)** *F. spetsiotica* × *F. rhodocanakis* ($2n = 3x$); **J)** *F. sporadum*. Scale bars = 10 μm.

these taxa have shown that *F. ehrhartii* has one acrocentric and one subtelocentric chromosome pair with small double satellites, whilst *F. sporadum* has two subtelocentric satellited chromosomes and two acrocentric chromosomes with secondary constriction (Kamari 1984b; Samaropoulou 2021, unpublished).

Likewise, the taxonomy of *Fritillaria pontica* Wahlenb. and *F. theophrasti* Kamari & Phitos has also been brought into question (Dimopoulos *et al.* 2013; Strid 2016). The aforementioned taxa differ in terms of morphology (Candargy 1897; Kamari & Phitos 2000; Samaropoulou 2021 unpublished), karyotype formula (Zaharof 1989; Bâsak 1991; Kamari & Phitos 2000; Samaropoulou 2021, unpublished), and seed and pollen morphology (Samaropoulou *et al.* 2019b, 2021). As regards the findings of silver nitrate staining, the maximum number of nucleoli was observed for both taxa in the current study (8 nucleoli). However, approximately 41% and 30% of the observed nuclei of *F. pontica* have 5 and 4 nucleoli, respectively (Fig. 3C), while 29% of the nuclei of *F. theophrasti* have 2 nucleoli, followed by 25% with 4 nucleoli (Fig. 4D). In addition, the nucleolar number of both taxa is greater than the number of secondary constrictions (Kamari & Phitos 2000; Samaropoulou 2021, unpublished), which may be easily explained since, despite the observation of several secondary constrictions, their number and presence is not always stable so as to define marker chromosomes (Table 1).

Two endemic subspecies with isolated distribution are *F. obliqua* subsp. *obliqua* Ker-Gawler (Figs. 1E & 3A), distributed in Sterea Hellas (Attika) and C & S parts of the island of Evia, and *F. obliqua* subsp. *tuntasia* (Heldr. ex Halácsy) Kamari (Figs. 1F & 3B), which is found in some of the northern Cyclades islands (Gyaros, Kythnos, Serifos). The similarity in the number of nucleoli between those two taxa (Table 1) is one more feature in addition to their morphological and karyological similarity which serves to confirm their taxonomy at least

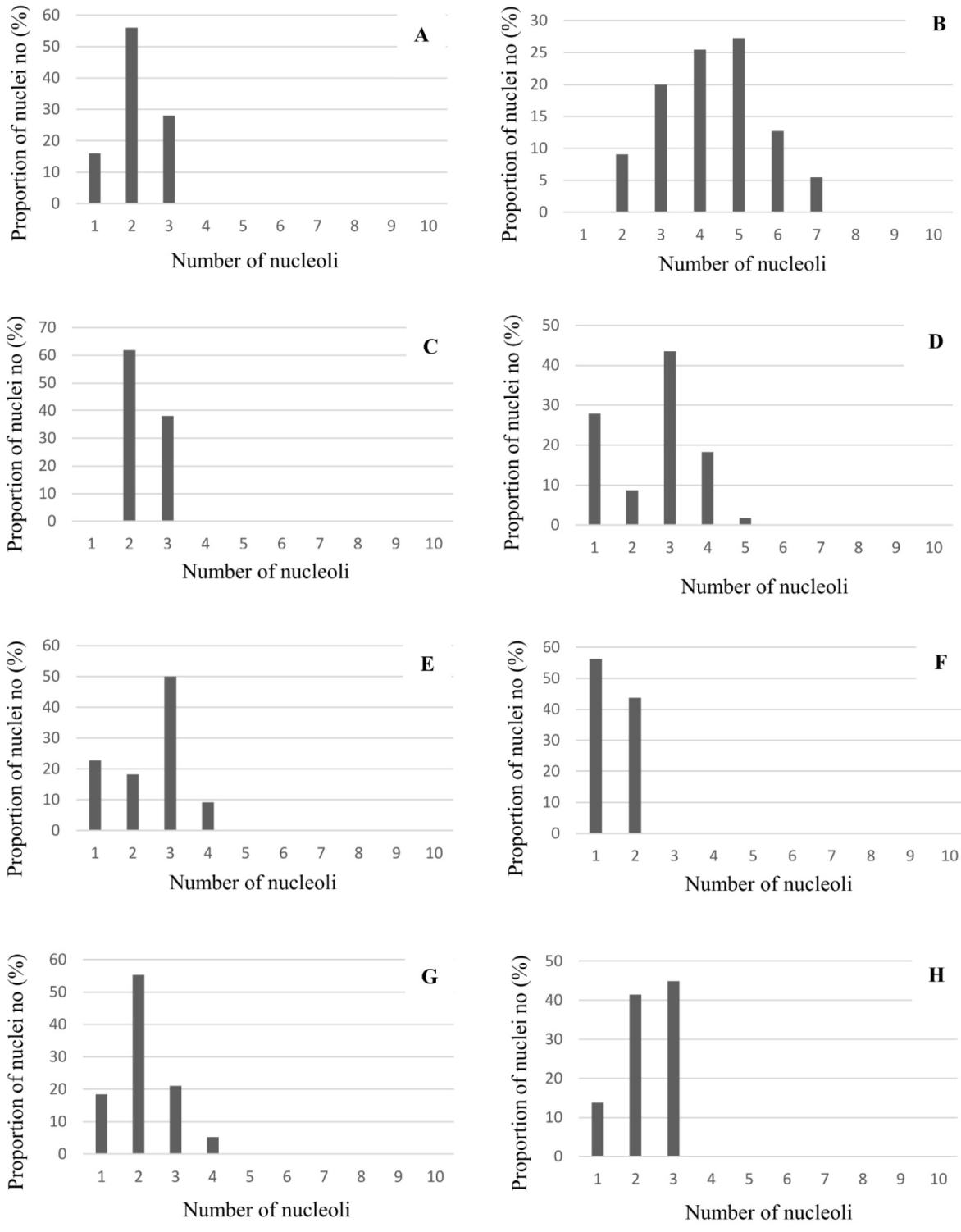

Fig. 2. Histograms of proportional frequency in nucleolar number. **A)** *F. ehrhartii*; **B)** *F. elwesii*; **C)** *F. graeca*; **D)** *F. messanensis* subsp. *messanensis* (SF1084); **E)** *F. messanensis* subsp. *messanensis* (F4); **F)** *F. messanensis* subsp. *gracilis*; **G)** *F. messanensis* subsp. *sphaciotica*; **H)** *F. mutabilis*.

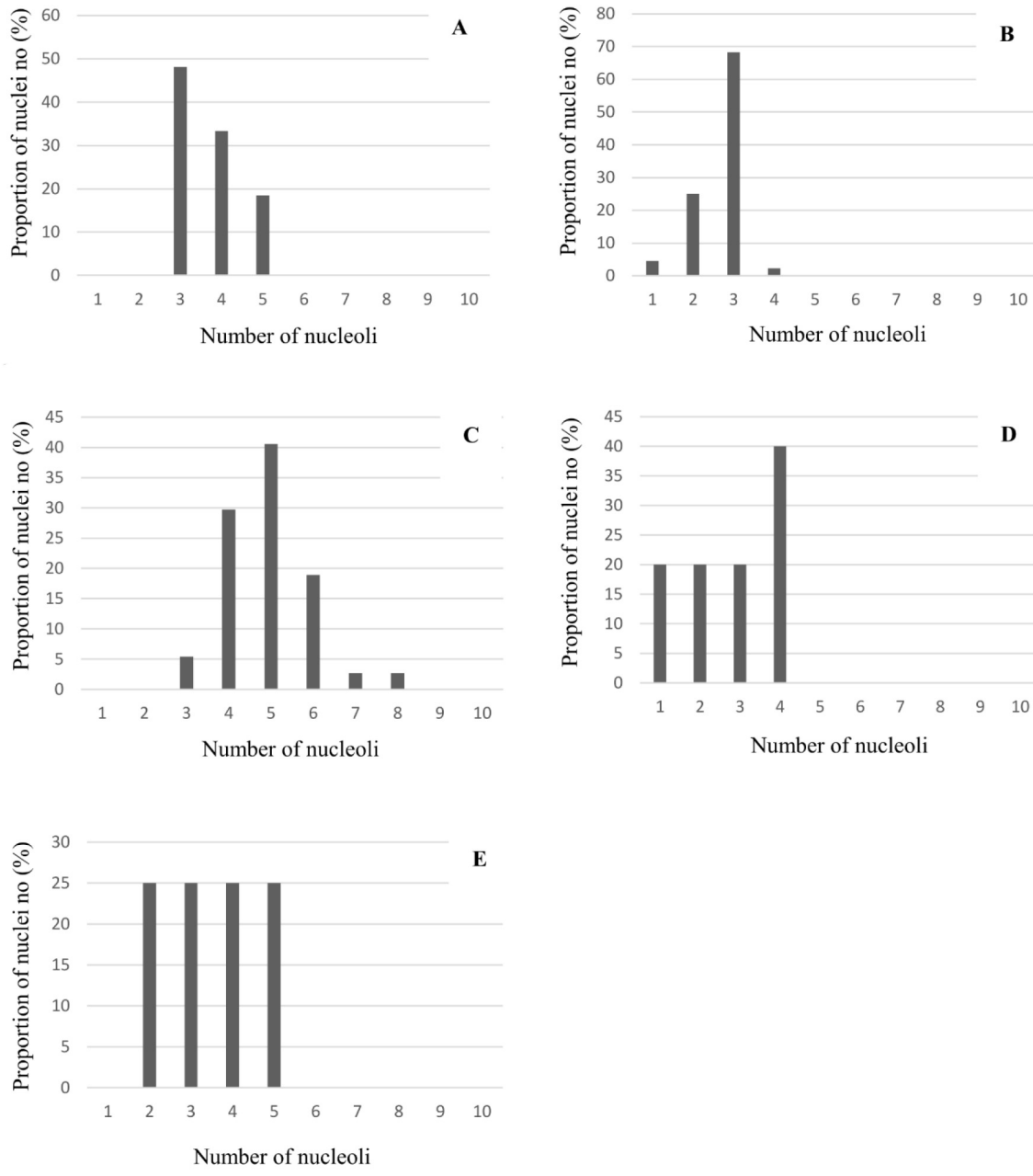

Fig. 3. Histograms of proportional frequency in nucleolar number. **A)** *F. obliqua* subsp. *obliqua*; **B)** *F. obliqua* subsp. *tuntasia*; **C)** *F. pontica*; **D)** *F. rhodocanakis*; **E)** *F. spetsiotica*.

up to subspecies level (Kamari 1984a, 1991a, 2009a; Kamari *et al.* 2009; Samaropoulou *et al.* 2019b).

Fritillaria messanensis Raf. is represented in Greece by three subspecies (*F. messanensis* subsp. *messanensis* Rafin., *F. messanensis* subsp. *gracilis* (Ebel) Rix and *F. messanensis* subsp. *sphaciotica* (Gand.) Kamari & Phitos. The maximum number of nucleoli observed in these typical subspecies of *F. messanensis* differs between the two populations studied (Fig. 2D & E). This observation is in accordance with previous reports mentioning differences in the number of satellite chromosomes between individual karyotypes from these two populations (Zaharof 1989; Kamari & Phitos 2006), which may be attributed to differences in altitude from sea level (Peloponnisos) to up to 2000 m (Mt. Olympus).

Fritillaria mutabilis Kamari has a similar number (Table 1) of nucleoli to *F. graeca* Boiss. & Spruner (Fig. 1B). This observation can be explained by the considered origin of *F. mutabilis* as a hybrid between *F. graeca* and *F. ionica* Halácsy s.l. (Kamari 1991a).

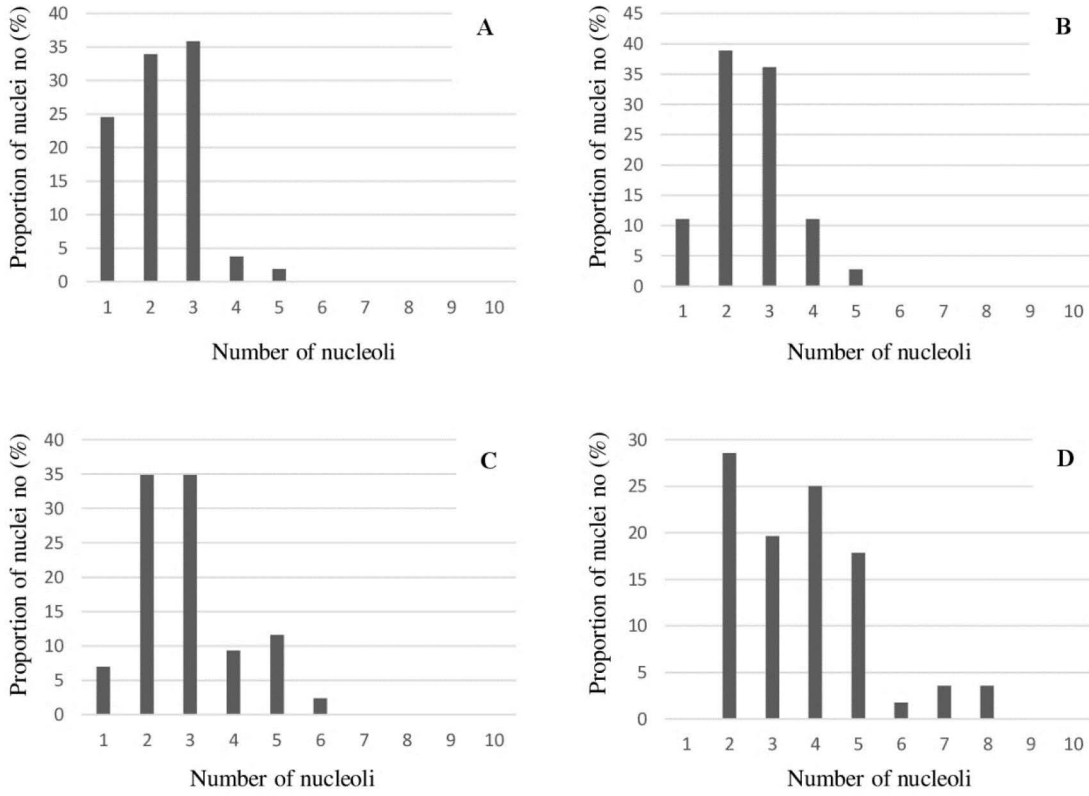

Fig. 4. Histograms of proportional frequency in nucleolar number. **A)** *F. spetsiotica* × *F. rhodocanakis* (2*n* = 2*x*); **B)** *F. spetsiotica* × *F. rhodocanakis* (2*n* = 3*x*); **C)** *F. sporadum*; **D)** *F. theophrasti*.

Fritillaria spetsiotica Kamari and *F. rhodocanakis* Orph., two stenoendemic taxa mainly distributed on the islands of the Argosaronic Gulf, tend to hybridise on the Argolis Peninsula where they co-exist. Even though they differ significantly in terms of the features of the flowers and leaves (colour and shape), they share similar karyotype morphology, a feature which may contribute to their ability to hybridise (Kamari 2009b; Kamari & Phitos 2009; Samaropoulou *et al.* 2019a). The number of their nucleoli was also similar, although *F. spetsiotica* was observed with a greater range of nucleoli compared to *F. rhodocanakis* (Table 1). In addition, the hybrids studied here also share a similar number of nucleoli (Fig. 4A & B). Although they differ in their ploidy level (2*x* and 3*x* respectively), triploid individuals are characterised by the presence of 0-2 euchromocatic B-chromosomes, which often have primary, as well as secondary constrictions (Samaropoulou *et al.* 2019a).

CONCLUSION

Fritillaria taxa present a variety of morphological and karyological features, indicating that the genus is still evolving, leading to taxonomical questions. Thus, despite the large number of studies carried out, the genus remains taxonomically very interesting and demands that different aspects be examined in order to provide more reliable conclusions about its taxonomy and phylogeny. The determination of the number of nucleoli and frequency of active NORs was made for the first time in 14 Greek representatives and one hybrid of different ploidy levels from Greece, as part of a general attempt to contribute to the genus' taxonomy and to resolve intra- and interspecific relationships. The results of the present study lead to conclusions which confirm previous research on the systematics, evolution and taxonomy of the genus *Fritillaria*.

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REZIME

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Variranje u broju nukleolusa kod *Fritillaria* (Liliaceae) taksona iz Grčke

Sofia Samaropoulou, Pepy Bareka, Aggelos Kanas i Georgia Kamari

Studija ima za cilj da proceni najčešći broj nukleolusa u taksonomski interesantnim taksonima *Fritillaria*. Kod 14 taksona i jednom hibridu iz 17 populacija iz Grčke primenjen je modifikovani protokol sa bojenjem srebro-nitratom. Ovo je prvi put da su kod taksona iz roda *Fritillaria* istraživani brojevi nukleolusa. Generalno, broj nukleolusa varira od 0 do 8, sa najvećim brojem konstatovanim kod vrsta *Fritillaria pontica* i *F. theophrasti*. Rezultati potvrđuju klasifikaciju taksonomski intrigantnih taksona, kao što su *Fritillaria sporadum* i *F. theophrasti*, kao različitih vrsta, iako se poslednje vreme smatraju sinonimima za *F. ehrhartii* i *F. pontica*, respektivno.

Ključne reči: kariologija, nukleolusi, $AgNO_3$, NORs, grčki endemiti