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Female Gametophyte Development of *Fritillaria stribrnyi* Velen. (Liliaceae), the Threatened Species in Southeast Europe

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Summary *Fritillaria stribrnyi* Velen. (Liliaceae) grows naturally only in northwest Turkey (Turkish Thrace) and southern Bulgaria, and is threatened with extinction in the wild due to human pressure. In this paper, the megasporogenesis, megagametogenesis, and embryo sac development of *F. stribrnyi* were investigated using light microscopy. Buds and blooming flowers were collected from the plant's natural habitats in the Ipsala district (Edirne, Turkey) from February to April of 2015 and fixed in ethanol: glacial acetic acid solution. Conventional paraffin embedding techniques were followed. Serial sections of the ovaries cut at 10–12 μm thickness were stained with alcian blue and nuclear fast red double staining. The ovary of *F. stribrnyi* is trilocular. The mature ovule is anatropous, tenuinucellate, and bitegmic. The archesporial cell differentiates directly into the megaspore mother cell. The megaspore mother cell undergoes regular meiotic division and a typical diagonal megaspore tetrad eventually form. The embryo sac of the species is eight-nucleated, a tetrasporic type, and followed the Fritillaria-type development. The egg cell and synergid cells are covered by a complete cellulosic wall in the mature embryo sac. Two of the antipodal cells degenerate before fertilization and the third cell shows a flattened structure. A haploid polar nucleus at the micropyle side comes with the second male nucleus toward the triploid one at the chalazal side after fertilization and triple fusion occurs.

Keywords *Fritillaria stribrnyi*, Liliaceae, Megasporogenesis, Megagametogenesis, Embryo sac, Fritillaria-type.

The genus *Fritillaria* L. is composed of seven subgenera and 140 species (165 taxa), which are distributed worldwide in the Northern Hemisphere, including Central Asia, Japan, China, North America, North Africa, Europe, and the Mediterranean region (Teksen and Aytac 2011). The genus is represented by 35 species (41 taxa), 17 of which are endemic, in the Turkish flora (Güner *et al.* 2012). The diversity center of the *Fritillaria* genus is the Mediterranean region (Tomovic *et al.* 2007). Thus, Turkey is very rich in both the total number of species and the number of endemic species. Some species, which grow wild, are cultivated because of their elegant flowers (*F. imperialis*, *F. persica*, *F. meleagris*, *etc.*, Ulug *et al.* 2010). In addition to being an ornamental plant, *Fritillaria* species such as *F. cirrhosa*, *F. delavayi*, *F. przewalskii*, *F. thunbergii*, *F. unibracteata*, and *F. verticillata* have been used as important drugs in traditional Chinese medicine for thousands of years, for example, against coughs, as an expectorant, and to relieve high blood pressure. Around 200 *Fritillaria* preparations are known (Hao *et al.* 2013).

The *Fritillaria* spp. have been studied for their morphological (Khaniki 2003, Teksen and Aytac 2011), karyological (Basak 1991, Kocyigit *et al.* 2016), palynological (Ozler and Pehlivan, 2007, Teksen *et al.* 2010, Shahla 2018), phylogenetic (Celebi *et al.* 2008, Advay and Sharifi-Tehrani 2016), phytochemical (Hao *et al.* 2013, Kiani *et al.* 2015) and molecular (Turktas *et al.* 2012) characteristics. In recent years, *in vitro* propagation and *in situ* and *ex situ* conservation research on rare, endemic, and economically important *Fritillaria* species has increased (Ulug *et al.* 2010, Kulkhanova *et al.* 2015, Muraseva *et al.* 2015, Shafi *et al.* 2018). Embryological studies carried out with the genus *Fritillaria* are rather limited. Studies on the development of the embryo sac in *F. pudica* (Sax 1916), *F. persica* (Bambacioni 1928), *F. liliacea* (Agrawal 1950), *F. tenella* (Davis 1966), and *F. ussuriensis* (Li and Shen 1990) have been reported. Recently, the development of the male gametophyte and *in vitro* pollen germination of *F. stribrnyi* has been investigated by Kartal (2015).

F. stribrnyi spreads in a limited area of northwest Turkey and southern Bulgaria. It is distributed naturally in Turkey only in the Turkish Thrace (Çanakkale, Edirne, Istanbul, and Kirklareli provinces; Rix 1984, Basak 1991). According to Petrova and Vladimirov (2009), the conservation status of the species in Bulgaria is critically endangered, whereas according to the Turkish Red Data Book (Ekim *et al.* 2000), it is endangered in

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Turkey.

In Turkey, a small number of *F. stribrnyi* individuals are observed in small populations and are faced with a very high risk of extinction due to human pressure on the environment. The species grows generally under *Paliurus spina-christi* (Christ's thorn) in pastures and meadows. The number of individuals in the habitat is rapidly decreasing due to the destruction of Christ's thorns in pastures. This work is part of an ongoing project on the reproduction and conservation biology of *F. stribrnyi*. The aim of the paper is to provide a comprehensive explanation of the megasporogenesis and development of the embryo sac of *F. stribrnyi*. The work will be continued with seed germination experiments, *in vitro* bulb micropropagation, and embryo rescue applications.

Materials and methods

Flower buds and blooming flowers of *F. stribrnyi* were collected from the plant's natural habitat in the pasture of Hidirkoy village of Ipsala district (40°54′48″N, 26°28′23″E, Edirne, Turkey), from 10:00 to 10:30 a.m. between February and April 2015. The specimens were fixed in the field in an ethanol–acetic acid mixture (3 : 1, v/v) and incubated in fixative at 20°C for 24 h. Then, the fixative was changed to 70% ethanol and the specimens were stored at 4°C until needed. Conventional dehydration, paraffin embedding, and microtome techniques defined by Jensen (1962) were applied. Serial sections of the ovaries, 10–12 μm thick, were cut using a Leica rotary microtome RM2255 (Nussloch, Germany). The sections were deparaffinized with xylene (Merck) overnight. Subsequently, they were passed through a gradually decreasing ethanol series (100 to 30%) for 10 min per change and rinsed in distilled water for 5 min. The sections were stained with 1% alcian blue (Sigma) for 45 min, washed with running tap water for 2 min, and rinsed with distilled water. Afterward, they were stained in 0.1% nuclear fast red (Sigma) aqueous solution for 5 min. The slides were washed with running tap water for 2 min and rinsed with distilled water. They were dehydrated using an increasing ethanol series (30 to 100%) and passed through an ethanol-xylene series before mounting with Entellan (Kartal 2015). Slides were examined with an Olympus BH-2 light microscope (Tokyo, Japan), and selected images were taken using a ProgRess C12 digital camera (Jena, Germany).

Results

The gynoecium of *F. stribrnyi* is between 18–20 mm in length in the flowering stage. The ovary is trilocular, syncarpous, and superior, containing approximately two hundred ovules, which are axillary placed. The mature ovule is anatropous type, bitegmic, and tenuinucellate. First, the inner integument develops and then the outer integument begins to appear as a small protuberance. The outer integument usually consists of three layers of cells and the inner integument is composed of two cell layers. The micropyle is formed by the inner integument at the mature embryo sac stage, and the inner integument is three- to four-layered around the micropyle.

The nucellar tissue has only one archesporial cell, which differentiates directly into the megaspore mother cell (Fig. 1a). The megasporocyte undergoes meiosis I, including leptotene (Fig. 1b), zygotene (Fig. 1c), pachytene (Fig. 1d), diplotene (Fig. 1e), diakinesis (Fig. 1f), metaphase I (Fig. 1g), anaphase I, and telophase I (Fig. 1h) and finally forming a dyad. The stages of meiosis I occur regularly in the megaspore mother cell. During the diplotene stage, the megasporocyte starts to increase in size (Fig. 1e). Following the first meiotic division, one daughter nucleus migrates to the micropylar side and the other migrates to the chalazal side, without the formation of a wall between them. The second meiotic division produces four free megaspore nuclei in the same cytoplasm, which have a typical zigzag arrangement depending on the breadth of the embryo sac (Fig. 1i–j). The embryo sac is tetrasporic and eight-nucleated, and embryo sac development follows that of the Fritillaria-type.

At the beginning of megagametogenesis, one of four megaspore nuclei is located at the micropylar end, and the other three nuclei move to the chalazal side. Thus, the four megaspore nuclei are polarized, with one toward the micropylar side and the other three toward the chalazal side (Fig. 2a). During the first embryo sac mitotic division, three chalazal spindles fuse to form a large spindle at metaphase (Fig. 2b), and the paired chromosomes separate at anaphase and telophase (Fig. 2c, d). At the end of the first mitotic division, a secondary four-nucleate stage occurs, with two large triploid nuclei at the chalazal side and two small haploid nuclei at the micropylar side. At this stage, the vacuole located in the central part of the embryo sac separates the micropylar and chalazal groups of nuclei. During the second mitotic division, this vacuole remains in the embryo sac (Fig. 2e, f). After the second division, there are eight nuclei and seven cells in the embryo sac. In this stage, the mature embryo sac includes the egg apparatus (an egg cell and two synergid cells), the haploid polar nucleus at the micropylar side, and a triploid polar nucleus, generally, three triploid antipodal cells at the chalazal side (Fig. 2g). In 76% of the occurring embryo sacs, two consecutive normal mitotic divisions are observed in the cells on the chalazal side and three antipodal cells and a polar nucleus are formed. In 7% of the embryo sacs, on the other hand, the lowest of the two nuclei on the chalazal side are observed to fail to undergo mitotic division, and a polar nucleus and two antipodal cells are formed. During the second mitotic division, the nucleus that will give the polar nucleus and one antipodal cell normally divide, but other cell division abnormalities, such as telophase

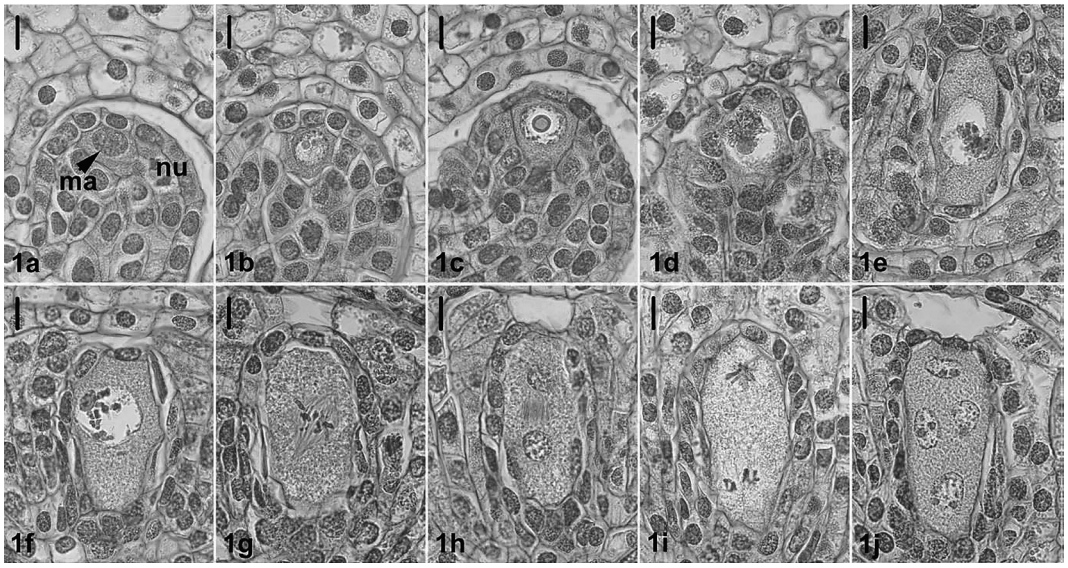


Fig. 1. Microsporogenesis in *F. stribrnyi*. (a) Megaspore mother cell; (b) leptotene stage; (c) zygotene stage; (d) pachytene stage; (e) diplotene stage; (f) diakinesis stage; (g) metaphase I stage; (h) telophase I stage; (i) metaphase II stage; (j) four haploid megaspore nuclei. ma: megaspore mother cell; nu: nucellus. Scale bars=20 μm.

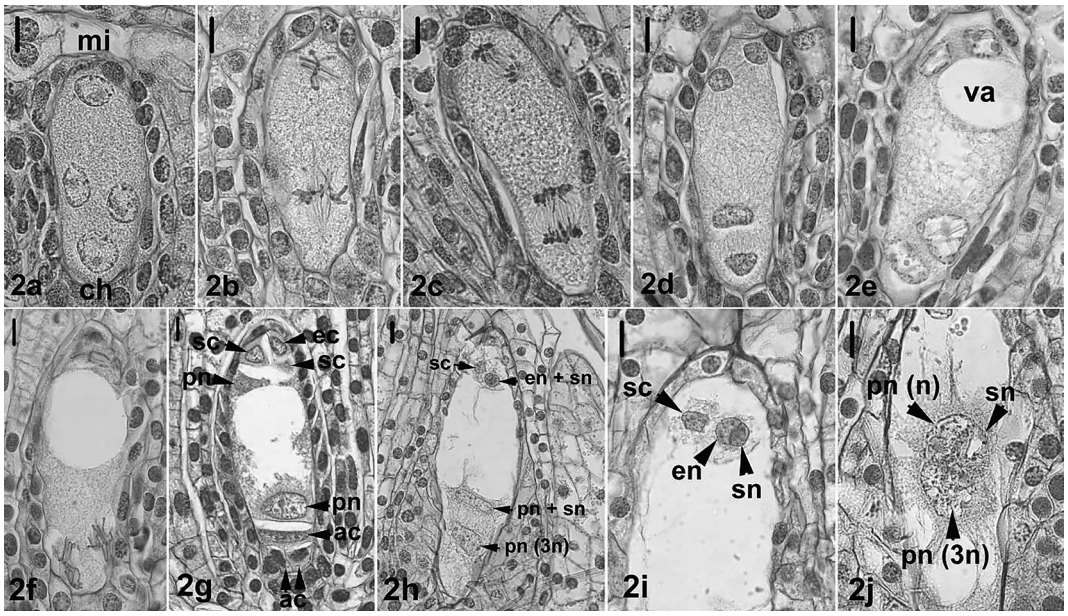


Fig. 2. Megagametogenesis and fertilization in *F. stribrnyi*. (a) four megaspore nuclei are polarized in a 1+3 distribution toward the micropylar side and chalazal side, respectively; (b) metaphase I stage; (c) anaphase I stage; (d) telophase I stage; (e) prophase II stage; (f) metaphase II stage; (g) mature embryo sac; (h) double fertilization; (i) syngamy (fusion of sperm and egg nuclei); (j) triple fusion (fusion of two polar nuclei and a sperm nucleus). ac: antipodal cell; ch: chalazal side; ec: egg cell; en: egg nucleus; mi: micropylar side; pn: polar nucleus; sc: synergid cell; sn: sperm nucleus; va: vacuole. Scale bars=20 μm.

bridges and chromosome disorders, are observed in 17% of the embryo sacs, leading to mitosis failures.

In the mature embryo sac, the egg cell and synergid cells are covered by a complete cellulosic wall. The central cell contains less peripheral cytoplasm, and a large vacuole separates the two polar nuclei at the micropylar side (haploid) and chalazal side (triploid) in the central cell (Fig. 2g). The antipodal cells are also surrounded by a complete cell wall. Two of these cells degenerate before fertilization, and the third cell becomes flat (Fig. 2g). During fertilization, one of the synergids is destroyed by the entry of the pollen tube through the micropyle. The second synergid remains intact for some period after fertilization and degenerates gradually. One of the male nuclei that enter the embryo sac goes toward the egg nucleus. The male and egg nuclei come into close contact but do not fuse (Fig. 2h, i). The other male nucleus moves toward the haploid polar nucleus. This male nucleus first comes into contact with the polar nucleus on the micropylar end, and these nuclei then

migrate toward the triploid chalazal polar nucleus (Fig. 2h). The polar nuclei and male nucleus remain in contact without fusing for a considerable time (Fig. 2j). Finally, they fuse completely, resulting in a pentaploid primary endosperm nucleus. The female and male nuclei then fuse to form a zygote.

Discussion

This study described the development of the female gametophyte of *F. stribrnyi*, which faces a very high risk of extinction caused by human pressure on the environment. In common with other members of the family Liliaceae, *F. stribrnyi* has a trilocular, syncarpous ovary and numerous ovules in each carpel (Davis 1966). The ovules of *F. stribrnyi* are anatropous, bitegmic, and tenuinucellate, and the embryo sac development is the same as that observed in other *Fritillaria*-type species, such as *F. liliacea, F. pudica, F. tenella*, and *F. ussuriensis* (Sax 1916, Agrawal 1950, Davis 1966, Li and Shen 1990). In *F. stribrnyi*, the archesporial cell develops directly into the megaspore mother cell, as found in many Liliaceae members that have been studied, including *F. liliacea, Gagea fascicularis*, *G. chrysantha, G. bohemica, G. granatellii*, *G. villosa, Lilium philippinense*, and *L. henryi* (Cooper 1935, Santos 1937, Joshi 1946, Agrawal 1950, Davis 1966, Caparelli *et al.* 2006, Vardar *et al.* 2012, Ekici 2014). All stages of megasporogenesis proceed as normal, and four haploid megaspore nuclei, arranged diagonally, are formed at the end of the second meiotic division for *F. stribrnyi*. Although the megaspores are arranged in a row in *L. henryi* (Cooper 1935) and *L. candidum* (Mikulska and Rodkiewicz 1965), they are seen in both linear and zigzag forms in *F. liliacea, G. chrysantha, G. granatellii* (Caparelli *et al.* 2006), and *G. bohemica* (Caparelli *et al.* 2006, Vardar *et al.* 2012). Ekici (2014) has reported that the embryo sac development in *G. villosa* is of the bisporic Endymion type.

The micropylar nucleus remains at the micropylar end of the embryo sac, and the other three nuclei are drawn to the chalazal side in *F. stribrnyi*. This 1+3 distribution is also seen in other Liliaceae members with Fritillaria-type embryo sac development (Sax 1916, Cooper 1935, Santos 1937, Joshi 1946, Agrawal 1950, Mikulska and Rodkiewicz 1965, Li and Shen 1990, Caparelli *et al.* 2006, Vardar *et al.* 2012). The megaspore at the micropylar end undergoes two consecutive mitotic divisions, resulting in four haploid nuclei without cytokinesis. Three haploid nuclei differentiate into the egg apparatus (one egg cell and two synergid cells), and one of them forms the polar nucleus in *F. stribrnyi*, much like in *F. pudica* (Sax 1916), *F. liliacea* (Agrawal 1950), and *F. ussuriensis* (Li and Shen 1990). At the chalazal side, the chromatins of the three nuclei undergo condensation and their membranes disappear. The chromosome groups are then placed on a common equatorial plate and enter mitotic division. At the end of two mitotic divisions, four triploid nuclei are formed in *F. stribrnyi*. These nuclei develop as a polar nucleus and three antipodal cells in 76% of the embryo sacs (Fig. 2g). Although there are usually three antipodal cells, two cells may be present at a rate of 7%, and division abnormalities may be found at a rate of 17%. As a result, the mature embryo sac generally has seven cells and eight nuclei, as a haploid egg cell, two synergid cells, and a polar nucleus (in the central cell) at the micropylar end and a triploid polar nucleus (in the central cell) and three antipodal cells at the chalazal side. Two of the antipodal cells are smaller and irregularly shaped compared with the other antipodal cell. *F. pudica* also contains seven-nucleate mature embryo sacs because the lowest of the chalazal nuclei fails to divide during the second mitotic division (Sax 1916). Agrawal (1950) has reported that the nearest nucleus at the chalazal side fails to divide and therefore develops the embryo sac with seven nuclei in *F. liliacea*. *F. ussuriensis* has seven-nucleated embryo sacs due to the degeneration of one of the chalazal nuclei (Li and Shen 1990). Cooper (1935) reported that in *L. henryi*, the nucleus nearest the chalazal side goes into mitotic division simultaneously with the other three normal developing nuclei in the embryo sac and gives two nuclei, both irregular in shape. Eventually, the embryo sac of *L. henryi* has eight nuclei. In a study on *L. philippinense*, Santos (1937) reported that four nuclei proceeded to a second mitotic division to form eight nuclei, but the lowest nucleus of the chalazal side was in the process of degeneration. Joshi (1946) also reported that the mature embryo sac of *G. fascicularis* contains seven nuclei and that one of the chalazal nuclei failed to divide during the second mitotic division. Although *G. chrysantha* and *G. bohemica* have eight-nucleated and seven-nucleated embryo sacs, respectively, *G. granatellii* has only seven-nucleated embryo sacs (Caparelli *et al.* 2006, Vardar *et al.* 2012).

During fertilization, one of the male gametes contacts the egg nucleus, and their contact points are flattened. The male and female gamete nuclei remain that way for a long time, eventually merging to form the zygote. The second male gamete contacts the haploid polar nucleus at the micropylar end, and the two nuclei migrate together near the triploid polar nucleus at the chalazal side. Triple fusion occurs, and ultimately the pentaploid primary endosperm nucleus is formed. These results have been reported previously in *F. pudica* and *F. ussuriensis* (Sax 1916, Li and Shen 1990). The present study detailed the embryological characteristics of *F. stribrnyi*, a threatened species that is facing a very high risk of extinction in the wild due to human pressure.

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References

Advay, M. and Sharifi-Tehrani, M. 2016. Taxonomic relationships of ten *Fritillaria* species of subgenera *Fritillaria* and *Theresia* based on analysis of flower qualitative and quantitative morphological characters. J. Genet. Resour. **2**: 10–20.

Agrawal, J. S. 1950. The embryo sac of *Fritillaria liliacea.* Proc. Natl. Acad. Sci. India **16**: 87–92.

Bambacioni, V. 1928. Ricerche sulla ecologia e sulla embriologia di "*Fritillaria persica*". L. Ann. Bot. (Roma) **18**: 7–37.

Basak, N. 1991. The genus *Fritillaria* (Liliaceae) in European Turkey. Bot. Chron. **10**: 841–844.

Caparelli, K. F., Peruzzi, L. and Cesca, G. 2006. A comparative analysis of embryo-sac development in three closely-related *Gagea* species (Liliaceae), with some considerations on their reproductive strategies. Plant Biosyst. **140**: 115–122.

Celebi, A., Teksen, M., Acik, L. and Aytac, Z. 2008. Taxonomic relationships in genus *Fritillaria* (Liliaceae): Evidence from RAPD-PCR and SDS-PAGE of seed proteins. Acta Bot. Hung. **50**: 325–343.

Cooper, D. C. 1935. Macrosporogenesis and development of the embryo sac of *Lilium henryi.* Bot. Gaz. **97**: 346–355.

Davis, G. L. 1966. Systematic Embryology of the Angiosperm. John Wiley Sons Inc., London.

Ekici, N. 2014. Development of female gametophyte in *Gagea villosa.* Cytologia **79**: 69–77.

Ekim, T., Koyuncu, M., Vural, M., Duman, H., Aytac, Z. and Adıgüzel, N. 2000. Red Data Book of Turkish Plants (Pteridophyta and Spermatophyta). Turkish Association for the Conservation of Nature-Van Centennial Univ. Barıscan Ofset, Ankara (*In Turkish*).

Güner, A., Aslan, S., Ekim, T., Vural, M. and Babac, M. T. (eds.) 2012. A Checklist of the Flora Turkey (Vascular Plants). Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını, İstanbul (*In Turkish*).

Hao, D. C., Gu, X. J., Xiao, P. G. and Peng, Y. 2013. Phytochemical and biological research of *Fritillaria* medicine resources. Chin. J. Nat. Med. **11**: 330–344.

Jensen, W. A. 1962. Botanical Histochemistry: Principles and Practice. W.H. Freeman and Company, London.

Joshi, A. C. 1946. Development of the embryo-sac of *Gagea fascicularis* Salisb. Bull. Torrey Bot. Club **67**: 155–158.

Kartal, C. 2015. Microsporogenesis, microgametogenesis and *in vitro* pollen germination in the endangered species *Fritillaria stribrnyi* (Liliaceae). Caryologia **68**: 36–43.

Khaniki, G. B. 2003. Fruit and seed morphology in Iranian species of *Fritillaria* subgenus *Fritillaria* (Liliaceae). Pak. J. Bot. **35**: 313–322.

Kiani, M., Sefidkonb, F., Babaei, A. and Naghavi, M. R. 2015. Phytochemical profiling of medicinal isosteroidal alkaloids of Iranian *Fritillaria* spp. (Liliaceae). Ind. Crops Prod. **70**: 451–458.

Kocyigit, M., Rastgeldi, U. and Kaya, E. 2016. Cytotaxonomical analysis of eleven Turkish *Fritillaria* L. (Liliaceae) taxa. IUFS J. Biol. **75**: 19–28.

Kulkhanova, D. S., Erst, A. A. and Novikova, T. I. 2015. *In vitro* regeneration from bulbous scales of *Fritillaria sonnikovae*, an endemic species. Russ. J. Dev. Biol. **46**: 215–221.

Li, Y. F. and Shen, J. H. 1990. The Studies on embryology in *Fritillaria ussuriensis* Maxim. Acta Bot. Sin. **32**: 499–504.

Mikulska, E. and Rodkiewicz, B. 1965. Fine structure of a developing embryo sac of *Lilium candidum.* Flora **155**: 586–595.

Muraseva, D. S., Novikova, T. I. and Erst, A. A. 2015. *In vitro* propagation and conservation of rare species *Fritillaria meleagris* L. from floral explants. Contemp. Probl. Ecol. **8**: 754–763.

Petrova, A. and Vladimirov, V. (eds.) 2009. Red list of Bulgarian vascular plants. Phytol. Balc. **15**: 63–94.

Ozler, H. and Pehlivan, S. 2007. Comparison of pollen morphological structures of some taxa belonging to *Asparagus* L. and *Fritillaria* L. (Liliaceae) from Turkey. Bangladesh J. Bot. **36**: 111–120.

Rix, E. M. 1984. *Fritillaria* L. In: Davis, P. H. (ed.). Flora of Turkey and the East Aegean Island. Vol. 8. Edinburgh Univ. Press, Edinburgh. pp. 284–302.

Santos, J. K. 1937. Macrosporogenesis of *Lilium philippinense.* Cytologia **41**: 822–835.

Sax, K. 1916. Fertilization in *Fritillaria pudica.* Bull. Torrey Bot. Club **43**: 505–522.

Shafi, H., Nawchoo, I. A., Shah, S. A. and Kaloo, Z. A. 2018. *Ex situ* conservation strategies for *Fritillaria cirrhosa* D. Don (Liliaceae): A critically endangered medicinal herb of Kashmir Himalaya. Proc. Natl. Acad. Sci. India **88**: 33–41.

Shahla, H. 2018. Pollen morphology of some *Fritillaria* L. species (Liliaceae) from Iran. Pak. J. Bot. **50**: 2311–2315.

Teksen, M. and Aytac, Z. 2011. The revision of the genus *Fritillaria* L. (Liliaceae) in the Mediterranean region (Turkey). Turk. J. Bot. **35**: 447–478.

Teksen, M., Aytac, Z. and Pınar, N. M. 2010. Pollen morphology of the genus *Fritillaria* L. (Liliaceae) in Turkey. Turk. J. Bot. **34**: 397–416.

Tomovic, G., Vukojicic, S., Niketic, M., Zlatkovic, B. and Stevanovic, V. 2007. *Fritillaria* (Liliaceae) in Serbia: Distribution, habitats and some taxonomic notes. Phytol. Balc. **13**: 359–370.

Turktas, M., Aslay, M., Kaya, E. and Ertugrul, F. 2012. Molecular characterization of phylogenetic relationships in *Fritillaria* species inferred from chloroplast trnL–trnF sequences. Turk. J. Biol. **36**: 552–560.

Ulug, B. V., Korkut, A. B., Sisman, E. E. and Ozyavuz, M. 2010. Research on propagation methods of Persian lily bulbs (*Fritillaria persica* Linn.) with various vegetative techniques. Pak. J. Bot. **42**: 2785–2792.

Vardar, F., Ismailoglu, I. and Unal, M. 2012. Embryological and cytological features of *Gagea bohemica* (Liliaceae). Turk. J. Bot. **36**: 462–472.