Embryology of Gymnospermium microrrhynchum (Berberidaceae)

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한계령풀의 생식기관 발생형태
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ABSTRACT: An intensive study of the embryology of Gymnospermium microrrhynchum was conducted to provide information regarding a discussion of the phylogenetic relationships of the genus, which is yet unstudied. Our results indicated that Gymnospermium is similar to other genera of Berberidaceae in terms of its embryological features. Nevertheless, newly reported and unique features are the well-developed endothelium and the undifferentiated seed coat type. Until the study of Gymnospermium, it may have been considered to be closer to Caulophyllum and Leontice in the tribe Leonticeae. These three genera share many morphological features as well as molecular similarities, by which they are kept in the same tribe, Leonticeae. However, very little detailed embryological data regarding these genera have been published thus far. Gymnospermium was characterized according to the basic type of anther wall formation as well as its glandular tapetum, successive cytokinesis in the microspore mother cell, two-celled mature pollen grains, anatropous and crassinucellate ovules with a nucellar cap, well-developed endothelium, its Polygonum type of embryo sac formation, its nuclear type of endosperm formation, and its undifferentiated seed coat type. In comparison with Nandina, there are many differences, such as the dehiscence of the anther, the cytokinesis in the microspore mother cells, the shape of the megaspore dyad, and the seed characteristics. Although we had no available detailed embryological information regarding Caulophyllum and Leontice, which are genera that are more closely related to Gymnospermium, we could deduce from the phylogenetic relationship that Gymnospermium, Caulophyllum, and Leontice are more closely related to each other than other genera of Berberidaceae on the basis of the seed characteristics.

Keywords: Berberidaceae, Caulophyllum, embryology, Gymnospermium microrrhynchum, Leontice, seed coat

적 요: 속간 계통유연관계 해석을 위한 정보를 제공하기 위하여 현까지 연구되지 않은 한계령풀의 생식기관 발생학적 형태 연구가 수행되었다. 연구결과 한계령풀의 생식기관 형태는 매자나무과의 다른 속들과 비슷한 점이 많았다. 그럼에도 불구하고, 내주피 내강벽의 발달, 비분화 종피 구조 등과 같은 새로운 생식기관 특징들이 밝혀졌다. 지금까지 계통학적으로 한계령풀은 매자나무과 내에서 형의다리아재비속이나 Leontice속에 가장 가깝다고 인정되고 있는데 이들이 같은 족에 분류되어 있는 것처럼 분자계통학 유사성과 더불어 많은 형태적 특징들도 공유하고 있었다. 한계령풀은 기본형의 악백형성, 분비형 용당세포, 좌분노세포의 연속형 세포질분열, 약 열개 2세포성 종세포화, 도생배주, 후충성주실, nucellar cap의 발달, 내주피 내강벽의 형성, 마디형 배낭형성, 횡형 배포형성, 미분화 종피 패턴 등의 특징을 나타내었다. 빼어 Nandina속과의 비교에서는 약 열개 패턴, 소포자의 세포질 분열패턴, 종파 형태, 종피 유형 등 많은 형태적 특징에서 차이가 있었다. 비록 이 연구에서 근연속인 형의다리아재비속이나 Leontice속의 생식기관 정보를 충분히 이용할 수는 없었지만, 한계령풀은 종피와 종피 형태를 근거로 매자나무과 내에서 형의다리아재비속과 Leontice속에 가장 가까운 유연관계임을 추론할 수 있었다.

주요어: 매자나무과, 식물발생학, 형의다리아재비, 한계령풀, Leontice, 종피

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Gymnospermium Spach is distributed in Eastern Europe and northeast Asia with eight species in the world (Mabberley, 2008). Out of eight species, G. microrrhynchum (S. Moore) Takht. is distributed in Korea and northeast China (Chang et al., 2004). This species is a polycarpic perennial herb which is restricted to altitudes of 800 meter high mountainous woodland habitats, typically Quercus mongolica Fisch dominated forests (Chang et al., 2004). As a result of ongoing habitat and population decline recently, it has been placed on the national endangered species list by the Ministry of Environment (1999) and the Korea Forestry Service (Lee et al., 1996). According to the IUCN red list categorization, the current global status of G. microrrhynchum is evaluated as Vulnerable (Chang et al., 2001).

Until now, this species has been classified as Leontice of Berberidioideae (Loconte and Estes, 1989). In flora of the USSR, however, Fedchenko (1937) recognized that Gymnospermium was included within Leontice. After several decades, Takhtajan reanalyzed the distinguishing characters of Gymnospermium and transferred six species including L. microrrhyncha from Leontice into Gymnospermium. Therefore, L. microrrhyncha has changed to G. microrrhynchum considering former name as a basionym (Takhtajan, 1970). In this study, we also followed Takhtajan’s taxonomic treatment.

On the other hand, several extensive studies on floral anatomy (Terabayashi, 1981; 1983a, b; 1985), palynology (Kosenko, 1980; Nowickie and Skvarla, 1981), serology (Jensen, 1973), and karyology (Kuroki, 1965, 1967) have been conducted to understand evolutionary relationships among the genera of the Berberidaceae. Nevertheless, there remains a controversial issue regarding relationships among these generic groups. On the Gymnospermium, Airy Shaw (1973), Meacham (1980), and Terabayashi (1985) always classified into same group, (Bongardia-Calopodium-Gymnospermium-Leontice). However, Loconte and Estes (1989) was recognized as Leontice except Bongardia on the basis of chromosome number. They also mentioned that the resemblance of Bongardia and Leontice is the result of convergent evolution.

Concerning the embryological studies of Berberidaceae, Sasri (1969) examined Berberis umbellata and Eddiae and Russel (1984) investigated the development of megagametophyte in Nandina domestica. Furness (2008) also reported on the subject of microsporogenesis in Berberidaceae. Although a few characteristics were reported in Cardophyllum (Terabayashi, 1983a) and Mahonia (Schnarf, 1931), there remains insufficient information for a comparative study within that family. In particular, Terabayashi (1981, 1983a, b, 1985) conducted several studies concerning floral anatomy on vasculature. However, he did not give sufficient information regarding embryological features. Therefore, we need much more information of embryological features from the genus level for a comparative study.

In this paper, we present the embryological features of the G. microrrhynchum in an effort to find useful characteristics for understanding the generic relationships within the family. Embryology has often provided good evidence for taxonomic relationships in flowering plants (Tobe, 1989). On the other hand, no embryological study was done until now in this species.

### Materials and Methods

For the embryological study, flower buds, open flowers, and fruits of G. microrrhynchum were collected from natural habitats in Gangwon province from 2003 to 2009. The collection data were presented in Table 1. Inflorescences were fixed in FAA (5 parts formalin: 5 parts glacial acetic acid: 90 parts 50% ethanol) and preserved in 50% ethanol. Several flower buds and open flowers were dehydrated through a t-butyl alcohol series and then embedded in paraplast with melting point 56 to 58°C for microtoming. Serial sections cut with a rotary microtome at 6 to 8 µm in thickness were stained with Heidenhain’s haematoxylin, Safranin and Fastgreen FCF, and mounted with Entellan. For study of young anther and hard seed coat, several young flower buds and mature seeds were dehydrated with ethanol series and embedded in Technovit 7100 resin. Embedded materials were sectioned with a disposable knife. Serial sections of 4 to 5 µm thickness were dried and stained with 0.1% Toluidine blue O. The stained slides were mounted with Entellan. The number of cells in mature pollen was counted by staining with 1%
acetocarmine (Tobe and Raven, 1984). All prepared slides with various stages were observed with BX-50 light microscope (Olympus Co., Japan). Photographs were taken using a digital camera system attached to microscope.

Terminology to describe the anther wall formation followed that proposed by Davis (1966) and seed coat terminology followed those proposed by Corner (1976) and Schmid (1986).

**Results**

**Anthers and microspores**

Flowers are bisexual with six sepals, six glandular petals, six stamens, and two ovules in a carpel (Fig. 1A). The anther is tetrasporangiate (Fig. 1B). The wall prior to maturation comprises basically five cell layers: an epidermis, an endothecium, two middle layers and a tapetum (Fig. 1C). The middle layers have a common histological origin with both the endothecium and the tapetum (Fig. 1C). Therefore, the wall formation conforms to the Basic type. The tapetum is glandular and its cells are two-nucleate (Fig. 1E). Meiosis in a microspore mother cell is accompanied by successive cytokinesis (Fig. 1D) and the resultant microspore tetrads are tetrahedral. During maturation, the middle layers are degenerated and the epidermal cells are crushed in an irregular shape, while the cells of the endothecium become enlarged. Eventually, the endothecium develops fibrous thickenings and the epidermis of anther wall collapses (Fig. 1F, G). Anther dehiscence takes place by valvate with each flap common to two microsporangia of the theca (Fig. 1G, H). Mature pollen grains are two-celled at the shed time (Fig. 1I).

**Megagametophyte and nucellus**

Two ovules are borne in a papery carpel (Fig. 1A). The ovule is anatropous (Fig. 1A, 3A) and crassincellate (Fig. 2B). The hypodermal archesporium is one-celled differentiating beneath the apical epidermis of nucellus (Fig. 2A). The archesporium cell periclinaly divides into the primary parietal cell and the primary sporogenous cell. The primary sporogenous cell undertakes meiosis as dyad (Fig. 2C), and linear tetrad (Fig. 2D). In the megaspore tetrads, the three upper megaspores degenerate, and the chalazal side megaspore becomes functional. The functional megaspore

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**Fig. 1.** Development of anther and microspore in *Gymnosporium microrhynchum*. A. Longitudinal section (LS) of flower; B. Transverse section (TS) of flower through anther; C. TS of young anther showing basic type wall development; D. Microspores in successive cytokinesis; E. TS of older microsporangium showing two nucleate tapetal cells; F. TS of older microsporangium showing fibrous endothecium; G. TS of valvate dehiscent anther; H. Stamens showing valvate opening; I. Two nucleate mature pollen. Abbreviations: ca, carpel; ent, endothecium; ep, epidermis; gc, generative cell; ml, middle layer; ov, ovule; pe, petal; pmc, pollen mother cell; st, stamen; t, tapetum; ve, vegetative cell. Scale bars: A, B=200 μm; C, D, F = 10 μm; E, I = 20 μm; G = 500 μm and H = 2 mm.
develops successively into a two- (Fig. 2E), four- (Fig. 2F), and eight-nucleate embryo sac (Fig. 2G). Thus the mode of embryo sac formation is of the Polygonum type. Consequently, an organized mature embryo sac has seven-celled and eight-nucleate with an egg cell, two synergids, two polar nuclei and three antipodal cells. Three antipodal cells are degenerated before or soon after fertilization.

During megagametogenesis, the apical dermal cells of the nucellus are more or less enlarged and divide periclinal to form a nucellar cap (Fig. 2C, H). Nucellar tissue disappears in the mature embryo sac (Fig. 2G). Starch grains are absent in mature embryo sac. Chalazal haustorium is developed (Fig. 2G, I). Neither hypostase nor obturator is differentiated during development of ovule and seed.

**Integuments**

The ovule is bitegmic (Fig. 2B, 3A, B). At the megaspore
mother cell stage, the inner integument is mostly two to three cell layers thick (Fig. 2B), and is not developed as multiplicative in later stage (Fig. 3A). On the other hand, the outer integument is initiated usually four to five cell layers thick (Fig. 2B), but later it becomes multiplicative into ten to twelve cell layers thick (Fig. 3A). The innermost layer of inner integument develops as endothelium, directly bordering on the growing embryo sac (Fig. 2F, G). The micropyle is formed by both the inner and the outer integuments (Fig. 3A). Neither the inner nor outer integument enters vascular bundles (Fig. 3B).

**Fertilization, endosperm and embryo**

Fertilization is porogamous. Endosperm formation is of the Nuclear type (Fig. 2J). In early stages of embryogenesis, the embryo sac has an aggregated free endosperm nucleus around a zygote or proembryo (Fig. 2J, 3A). After fertilization, the zygote forms the apical cell and the basal cell through the cell division. Consequently, proembryo (Fig. 2J) and straight mature embryo (Fig. 3E) is formed. In mature seeds, endosperm is copious, whereas the embryo is small and dicotyledonous with a short suspensor (Fig. 3E).

**Seed and seed coat**

Generally, this species has two ovules in a carpel before mature seed stage. But in mature seed time, the papery carpel wall is all removed and produces a naked mature seed similar to *Caulyphyllum*. The seed of *Gymnospernum* is globose and naked (Fig. 3D, F). It is albuminous and exarillate with a dicotyledonous, small, symmetrical straight embryo (Fig. 3E, F). The young seed coat comprises a multi-cell-layered testa; a one-cell-exotesta, a sixteen to eighteen or even more cells mesotesta and a one-cell-endotesta, and two to three cell-layers tegmen (Fig. 3A, B). In terms of seed coat type, it is an ‘undifferentiated seed coat’, because the specialized mechanical layer is absent and the entire seed coat layer is represented by thin walled parenchymatous cells. Testa is highly multiplicative (Fig 3C). Exotesta is single layered, cells are thin walled parenchymatous and some what elongated (Figs 3C, G). Mesotesta is wide and endotesta not as distinct as in immature seed. All three layers of tegmen are crushed. The mature seed coat thus comprises single layered exotesta, poorly differentiated one-layered endotesta and multi-layered mesotesta (Fig. 3F).

![Fig. 3. Seed and seed coat of *G. micrornichum*. A. young seed showing proembryo and nuclear endosperm; B. young seed showing outer and inner integuments; C. TS of young seed coat; D. Mature seed; E. LS of mature seed showing small embryo; F. magnified embryo part; G. TS of mature seed coat. Abbreviations: em, embryo; exts, exotesta; ii, inner integument; mi, micropyle; mts, mesotesta; oi, outer integument; pem, proembryo; sc, seed coat; tg, tegmen; tst, testa. Scale bars: A = 100 μm; B = 50 μm; C = 20 μm; D, E, F = 1 mm and G = 200 μm.](image-url)
Discussion

Comparison with Caulophyllum and Nandina

According to molecular data, Gymnospermium is closely related with Caulophyllum and Leontice (Kim et al., 2004). However, embryological features of Leontice are totally lacking until now and only a few embryological features of Caulophyllum were reported (Johri, 1992; Furness, 2008; Terabayashi, 1983a). Gymnospermium and Caulophyllum are closely related and share most of the characteristics, but reported embryological characteristics are not actually consistent with each other. Successive type of cytokinesis in pollen grain is found in Gymnospermium. However, it is probably a simultaneous type in Caulophyllum (Furness, 2008). Also, integumentary vascular bundle is reported in Caulophyllum which is not found in Gymnospermium. Similarly modified Pecpermia type embryo sac found in Caulophyllum (Johri, 1992), but we found eight nucleate Polygonum type embryo sac in Gymnospermium. Nevertheless, the seed coat structure of Gymnospermium resembles Caulophyllum (Heo et al., unpubl. data).

The phylogenetic position of Nandina has been the most controversial issue in the systematic of the Berberidaceae (Jensen, 1973; Cronquist, 1981). In most classification, the genus is either excluded from the family or positioned as a basal member (Meacham, 1980; Cronquist, 1981; Locoste and Estes, 1989). However, the most recent sequence data from gapD (Adachi et al., 1995), rbcL (Kim and Jensen, 1996), nadF (Kim et al., 2004) and nuclear ribosomal DNA ITS (Wang et al., 2007) suggested that Nandina is not basal but is related to Caulophyllum and sometimes basal (Kim and Jensen, 1998). We follow the later one classification by which Nandina is a sister to Caulophyllum robustum, C. thalictroides, Leontice leontopetalum, and Gymnospermium microrrhynchum clade (Wang et al., 2007) and compare embryological characteristics with Gymnospermium microrrhynchum. Although detailed information about anther and microspore, seed coat structure, and embryoogy of Nandina is still lacking, the common embryological features of Gymnospermium microrrhynchum and Nandina domestica are as follows: Anther tetraraporangiate; microspore tetrad tetrahedral; tapetum glandular; mature pollen grain two-celled; ovule anatropous, bitegmic and crassinucellate, micropyte formed by both integuments; megaspore tetrad linear; embryo sac formation Polygonum type; albuminous and exarillate seed.

Although they have many similar embryological features as above mentioned, Gymnospermium and Nandina clearly differ in some characteristics. For example, dehiscence of anther through valve in Gymnospermium, but it is by a longitudinal slit in Nandina; successive cytokinesis in microspore in Gymnospermium, but it is simultaneous in Nandina; dissimilar dyad is described in Nandina, chalazal cell of the dyad is twice as large as micropylar, but we did not find such case in Gymnospermium. Seed is globose and erect on stout funicule in Gymnospermium but not in Nandina. On the seed coat type, Nandina has endotegmic type which is very distinct character in Berberidaceae, whereas Gymnospermium and Caulophyllum have “undifferentiated” seed coat type (Locoste, 1993; Heo et al., unpubl. data).

Comparison with other Berberidaceae

Comparatively very little and fragmented embryological data of Berberidaceae has been reported (Johri et al., 1992; Edbai and Russell, 1984; Furness, 2008; Satari, 1969). Although some notable differences are also found with some genera of the family, the genus totally agrees with Berberidaceae on the basis of investigated reproductive morphological features. Multinuclear (as many as eight) tapetal cells becomes polyploid after the nuclear fusion in most of genera in the family. We found two nucleate tapetal cells in Gymnospermium. Johri (1992) reported that only outer integument takes part in formation of micropyte in most of the Berberidaceae, in contrary both integuments formed micropyte in Gymnospermium microrrhynchum and Nandina domestica (Edbai and Russell, 1984). Ovule is crassinucellate, archesporium cuts off a parietal cell, one to many parietal layers are formed and functional megaspore mother cell develops into linear tetrad after meiosis division, no parietal layer formed in Jeffersonia (Andrews, 1895) and Podophyllum (Lubliner, 1925), and chalazal megaspore develops into Polygonum type embryo sac. Well-developed integumentary tapetum (endothelium) observed in G. microrrhynchum is not mentioned yet in other members of Berberidaceae. Endosperm is absolutely nuclear type. Onagrace type of embryogeny is found in most of the genera of Berberidaceae, but we could not confirm embryogeny detailed in Gymnospermium. Solanace type of embryogeny and polycylobaly were also observed in Barbieris aristata whereas tricotyledonous embryo found in Podophyllum peltatum (Johri, 1992).

The seed is albuminous and exarillate. Exostelial seed coat described for Berberidaceae (Johri, 1992). However, we could not find any specialized mechanical layer in Gymnospermium seed coat. Therefore, we would like to refer to it as ‘undifferentiated seed coat’ rather than exostelial type. Testa in Berberidaceae not or slightly multiplicative, in Gymnospermium it is highly multiplicative and mesotesta represents as many as 15 to 20 cell layers. Tegmen in mature seed of Gymnospermium is crushed as in other species of Berberidaceae or represented by very thin single layer (Corner, 1976; Johri, 1992).

In conclusion, Gymnospermium obviously shares most of the embryological characteristics with other genera of the Berberidaceae.
We could not compare Gymnospermium with other members of the tribe Leonticeae, because no detailed embryological features of the tribe have been carried out. Molecular studies have been suggested that Gymnospermium is more closely allied with Leontice than Caulophyllum in the tribe and Nandina remains a sister group of Leontice-Caulophyllum-Gymnospermium clade which also denies the monotypic family or subfamily (Kim et al., 2004; Wang et al., 2007). The consensus of Nandina as monotypic family or subfamily of Berberidaceae was based largely on primitive morphological features found in the Nandina (Meacham, 1980; Loconte and Estes, 1989; Takhtajan, 1997). However, it is very difficult to come to a critical systematic conclusion by comparing available embryological features of Gymnospermium because other genera of Berberidaceae and detailed embryological features are still lacking. Nevertheless, we could deduce the phylogenetic relationship that Gymnospermium is more closely related with Caulophyllum and Leontice than other genera of Berberidaceae on the basis of seed and seed coat characteristics.

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Literature Cited


