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SYSTEMATIC POSITION OF *MIYAKEA INTEGRIFOLIA* MIYABE & TATEW. IN THE INFRAGENERIC CLASSIFICATION OF *PULSATILLA* (RANUNCULACEAE): PALYNOLOGICAL CHALLENGES

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Abstract

Miyakea integrifolia Miyabe and Tatew. was considered to be an endemic genus and species to Sakhalin Island, Russia, but the monotypic genus *Miyakea* was later subsumed into *Pulsatilla* as *P. integrifolia* (Miyabe & Tatew.) Vorosch. This taxonomic treatment has been supported by many botanists. At present, there are two opinions on the systematic position of *P. integrifolia* within the genus *Pulsatilla*: i) a separate monotypic subgenus *Miyakea* situated at the last position; ii) a close relative of *P. patens* and *P. vernalis* in ser. *Patentes* within the species-rich subgen. *Pulsatilla*. Since palynological evidence might be promising morphological traits for clarifying the systematic position of *Miyakea*, a table summarizing the pollen morphological traits within the genus *Pulsatilla* was prepared using the previous appropriate palynological reports. Hitherto, the morphology of *Miyakea* pollen supports the second opinion i.e., a close relative of *P. patens* and *P. vernalis*. Moreover, we pointed out the issues that should be focused on in future research on the pollen morphology of *Pulsatilla* s.l.

Introduction

Genus *Pulsatilla* Miller (Pasqueflowers), contains *ca*. 40 perennial species, belongs to the tribe Anemoneae DC., subfamily Ranunculoideae Hutch. in the family Ranunculaceae A.L.Juss. (Tamura, 1991, 1993, 1995). Although phylogenies based on molecular data indicated that *Pulsatilla* was embedded within *Anemone* s.l. (Hoot *et al*., 1994, 2012), all previous molecular phylogenetic studies agreed regarding the monophyly of the genus *Pulsatilla* (Sramkó *et al*., 2019); therefore, the genus *Pulsatilla* is retained here.

Miyakea integrifolia Miyabe and Tatew., a closely related taxon of *Pulsatilla*, was collected from the mountain range of the palaeozoic formation situated on the Sea of Okhotsk side of Sakhalin Island in 1934 and was first described as a new monotypic and endemic genus in 1935 (Miyabe and Tatewaki, 1935, 1937). The presence of coriaceous, simple, entire, and evergreen radical leaves with three prominent parallel veins on the underside was regarded as the most prominent generic feature of this taxon (Miyabe and Tatewaki, 1935, 1941).

Although the generic status had been maintained by Sugawara (1939), Czerepanov (1995), Starodubtsev (1995), Smirnov (2002), Barkalov and Taran (2004), Nishikawa (2008) and Eryemin *et al*. (2019). Voroshilov (1966) considered the genus *Miyakea* to be *Pulsatilla* and published the name *P. integrifolia* (Miyabe & Tatew.) Vorosch. This view was followed by Czerepanov (1973) and Voroshilov (1982); furthermore, Tamura (1991, 1995) and Grey-Wilson (2014, 2020) recognized this species as a separate monotypic subgenus *Miyakea* situated at the last position

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within the classification system of *Pulsatilla.* On the other hand, the most recent molecular phylogenetic study by Sramkó *et al*. (2019) showed that *M. integrifolia* is subsumed into the genus *Pulsatilla* but is considered to be a closely related sister species to *P. vernalis* in ser. *Patentes* of subgen. *Pulsatilla.* Thus, ser. *Patentes* has been composed of three *Pulsatilla* species: *P. integrifolia* [≡*Miyakea integrifolia*], *P. patens*, and *P. vernalis*. The inclusion of *P. integrifolia* and *P. vernalis* within ser. *Patentes* is an unexpected result, as no specialist of *Pulsatilla* has ever considered the species included in this clade to be related (Sramkó *et al*., 2019).

Several studies on the pollen morphology of *Pulsatilla* species have been conducted: Kumazawa (1936), Ikuse (1956, 2001), Huynh (1970), Nakamura (1980), and Wang *et al*. (1995) with light microscopy (LM); Clarke *et al*. (1991) and Baladehi *et al*. (2013) with LM and scanning electron microscopy (SEM); Nowicke and Skvarla (1995), Miyoshi *et al.* (2011), Fujiki *et al*. (2016), and Sarwar and Takahashi (2023) with SEM; Xi (1985) and PalDat (2023) with LM, SEM, and transmission electron microscopy (TEM). Palynological studies of *Miyakea integrifolia* [as *P. integrifolia*] have not been carried out except for the SEM micrograph shown by Nowicke and Skvarla (1995) in “Die Natürlichen Pflanzenfamilien” on the family Ranunculaceae (Hiepko, 1995).

A summary table of the palynological traits of *Pulsatilla* including *Miyakea integrifolia* was made from appropriate palynological references (Huynh, 1970; Xi, 1985; Clarke *et al*., 1991; Nowicke and Skvarla, 1995; Miyoshi *et al*., 2011; Baladehi *et al*., 2013; Fujiki *et al*., 2016; Sarwar and Takahashi, 2023; PalDat, 2023) following the classification system within the genus *Pulsatilla* by Sramkó *et al*. (2019) (Table 1). Descriptive terminology follows Punt *et al*. (1994, 2007) and Hesse *et al*. (2009).

An overview of pollen morphological traits in the infrageneric taxonomy of *Pulsatilla* by Sramkó *et al*. (2019)

The most recent DNA-based classification of *Pulsatilla* by Sramkó *et al*. (2019) is characterized by the following three main results; i) the genus was separated into three subgenera, *Kostyczewianae*, *Preonanthus* and *Pulsatilla*, ii) the monotypic subgen. *Kostyczewianae* consisting of only *P. kostyczewii* was basally placed in the classification, iii) three species (*P. integrifolia* [≡*Miyakea integrifolia*], *P. patens* s.l. and *P. vernalis*) formed the single ser. *Patentes* of sect. *Pulsatilla* within the species-rich third subgen. *Pulsatilla.*

The basally positioned subgen. *Kostyczewianae* in the Sramkó *et al*.’s system was characterized by 2- to 3-colpate pollen with small grain size below 30 µm in the longest axis (Table 1). The two apertures state is palynologically exceptional within the genus *Pulsatilla* and supports the subgeneric status, but further studies are needed because 2-colpate pollen was reported in only 40% of pollen grains in Xi (1985).

Within the recent DNA-based classification by Sramkó *et al*. (2019), most species examined in the second subgen. *Preonanthus* (including two sects. *Preonanthus* and *Preonanthopsis*) were characterized by having commonly pantocolpate pollen with a medium (30 µm or more but below 40 µm) grain size (Table 1). The palynological evidence would support possibly the unity of subgen. *Preonanthus.*

The species-rich third subgen. *Pulsatilla* (including three sects. *Tatewakianae*, *Pulsatilla*, and *Semicampanaria*) is characterized by the following three palynological groups; i) diploid species with medium- to large-sized 3-colpate pollen (sect. *Tatewakianae* [poorly studied], ser. *Patentes* of sect. *Pulsatilla*, and sect. *Semicampanaria* excluding ser. *Albanae*); ii) tetraploid species with large-sized 3-colpate pollen (ser. *Pulsatilla* of sect. *Pulsatilla*); iii) diploid species with medium-sized pantoporate pollen (ser. *Albanae* of sect. *Semicampanaria*) (Table 1).

The irregular and occasional appearance of pantocolpate pollen adding to the usual 3-colpate pollen was observed in *P. patens*, *P. vulgaris*, *P. halleri*, *P. chinensis*, and *P. dahurica* within the third subgen. *Pulsatilla* (Table 1). This palynological phenomenon indicates at least a similarity between the second subgen. *Preonanthus* and the species-rich third subgen. *Pulsatilla*.

The majority of the species (eight examined among the twelve species) comprising the last series *Albanae* within the third subgen. *Pulsatilla* was characterized by having the most specialized pantoporate pollen within the genus *Pulsatilla* (Table 1). The evolutionary trend of pollen types; from tricolpate through pantocolpate to pantoporate was postulated in *Pulsatilla* (Xi, 1985). Series *Albanae* could be distinguished from other series within the genus *Pulsatilla* in the pollen aperture traits (Huynh, 1970; Tamura, 1995). Monophyly and the last position of series *Albanae* in the classification of Sramkó *et al.* (2019) was well supported by the present palynological evidence, and the section-level separation of *Albanae* should be possibly considered.

Although Nowicke and Skvarla (1995) pointed out that the pollen morphology of Ranunculaceae has generally limited taxonomic values, palynological traits, in some cases, indicate homogeneity at the infrageneric level in the *Pulsatilla* classification by Sramkó *et al*. (2019).

Systematic affiliation of *Miyakea* based on the palynological traits and future challenges

At present, there are two opinions on the systematic position of *Miyakea* [*P. integrifolia*] within the genus *Pulsatilla*: i) a separate monotypic subgen. *Miyakea* situated at the last position mainly based on morphological and anatomical studies (Tamura, 1991, 1995; Grey-Wilson, 2014, 2020), ii) a close relative of *P. patens* and *P. vernalis* in ser. *Patentes* within the species-rich subgen. *Pulsatilla* mainly based on DNA and chromosome analyses (Sramkó *et al.*, 2019).

Miyakea pollen was first studied by Nowicke and Skvarla (1995) in SEM and they showed that 3-colpate aperture and supratectal microspinulate ornamentation with distinct tectal perforations. A tri-colpate aperture of *Miyakea* pollen is the same as in many species of subgen. *Pulsatilla* (excluding series *Albanae*) within *Pulsatilla*. Although exine ornamentation such as supratectal microspinules is the same character between *Pulsatilla* and *Miyakea*, this feature is common within the Ranunculaceae (44 of the 48 genera examined; Nowicke and Skvarla, 1995).

Nowicke and Skvarla (1995) suggested that elongated tectal perforations on the surface view of *Miyakea* pollen were regarded as characteristic palynological features distinguished from *Pulsatilla*. But the surface shape of tectal perforations of *Miyakea* [as *P. integrifolia*] in their SEM micrograph may not be evidently different from that of *P. patens* (Xi, 1985; Plate 3, fig. 4) and *P. vernalis* (Clarke *et al*., 1991; Plate 47, Fig. 2) within ser. *Patentes*. Therefore, the exine ornamentation of *Miyakea* pollen may not support positively the opinion with the separate monotypic subgen. *Miyakea* (Miyabe and Tatew.) Tamura.

As in Table 1, the presence of distinct tectal perforations in SEM micrographs in most *Pulsatilla* pollen was different from indistinct perforations on the pollen in some samples of *Pulsatilla*; *P. kostyczewii*, *P. patens*, *P. grandis* and *P. pratensis* and *P. cernua*. However, considering the different preparation methods and micrograph resolution between them, we did not consider the presence/absence (or distinct/indistinct) of tectal perforations as the taxonomic informative palynological character at the current research stage. To compare the exine ornamentation between species within *Pulsatilla*, it is necessary to thoroughly remove the surface

Table 1. Palynological characters of *Pulsatilla* species following the classification by Sramkó *et al.* (2019).

Taxa	Distr.$^{1)}$	Chromosome number (2n)$^{2)}$	Apertures	Grain size$^{3)}$	Exine ornamentation$^{4)}$	Palynological references
Genus *Pulsatilla* Miller						
I. Subgen. *Kostyczewianae*						
P. kostyczewii (Korsh.) Juz.	A	16	3-colpate/2 (c. 40 %) —3-colpate (c. 60 %)	S/S	—/ind. & und.	Huynh (1970)/Xi (1985)
II. Subgen. *Preonanthus*						
2-1. Sect. *Preonanthus*	[B, D, H]					
P. alpina (L.) Delarb.	H	16, 32	pantocolpate/pantocolpate	M/M	—/—	Huynh (1970)/Clarke *et al.* (1991)
P. alba Reichb.	H	16, 32	pantocolpate	M	—/dist.	Huynh (1970)/Clarke *et al.* (1991)
P. aurea (Somm. & Lev.) Juz.	D	16	pantocolpate	M	—	Huynh (1970)
P. magadanensis Khokhryakov & Vorosch.	B	16	—	—	—	—
P. scherfelii (Ullep.) Skalicky	H	16	3-colpate	—	—	https://hardiness.zone/plant/?sle=Pulsatilla&art=scherfelii
2-2. Sect. *Preonanthopsis*	[B, G]					
P. taraoi (Makino) Takeda	B	16	pantocolpate (90%) and 3-colpate (10%)	M	—	Huynh (1970)
P. nipponica (Takeda) Ohwi	B	16, 32	6−8-pantocolpate	S-M	dist.	Miyoshi *et al.* (2011), Fujiki *et al.* (2016)
P. occidentalis (Wats.) Freyn	G	16	3-colpate (typically; sometimes mixed with 20% pantocolpate-like grs.)	M	—	Huynh (1970)
P. sachalinensis H.Hara	B		—	—	—	—
III. Subgen. *Pulsatilla*						
3-1. Sect. *Tatewakianae*	[B]					
P. tatewakii Kudo	B	16	—	—	—	—
P. sugawarai Miyabe & Tatew.	B		—	—	—	—
P. ajanensis Regel & Tiling	B	16	3-colpate	M	—	Huynh (1970)

Table 1 contd.

Taxa	Distr.$^{1)}$	Chromosome number (2n)$^{2)}$	Apertures	Grain size$^{3)}$	Exine ornamentation$^{4)}$	Palynological references
3-2. Sect. *Pulsatilla*	[B, C, E, F, G, H]					
3-2-1. Ser. *Patentes*	[B, C, E, G, H]					
P. integrifolia (Miyabe & Tatew.) Vorosch.	B	16	3-colpate (with 30% anomalous grs.)	—	elongated dist.	Nowicke & Skvarla (1995)
P. patens (L.) Mill.	C, E, G	16, 32	3-colpate/3-colpate/3-colpate/3-colpate/3-colpate with 6-pantocolpate irregularly/3-colpate	L/L/M/ L/L/L	—/—/dist./— /?ind. /—	Huynh (1970)/Huynh (1970) as *P. flavescens* (Zucc.) Juz., *P. nuttalliana* (DC.) Bercht. & Presl ssp. *nuttalliana* and *P. nuttalliana* ssp. *multifida* (Pritz.) Aich. & Schwgl./Xi (1985)/Xi (1985) as var. *multifida* (Pritz.) S.H.Li & Y.H.Huang/Xi (1985) as *P. flavescens* (Zucc.) Jub./Clarke *et al.* (1991)
P. vernalis (L.) Mill.	H	16	3-colpate/3-colpate/3-colpate/3-colpate	L/M/M/ —	—/?dist./ dist./dist.	Huynh (1970)/Xi (1985)/Clarke *et al.* (1991)/PalDat (2023)
3-2-2. Ser. *Pulsatilla*	[F]					
P. vulgaris Mill.	F	32	3-colpate/3-colpate/ pantocolpate (together 3-colpate?)/3-colpate	L/S/M/ M	—/dist./dist. /dist.	Huynh (1970) incl. var. *germanica* (Blocki) Aich. & Schwgl./Xi (1985)/Clarke *et al.* (1991)/PalDat (2023) as *P. orenipontana*
P. grandis Wenderoth	E	32	3-colpate/3-colpate/3-colpate	L/L/L	—/?ind. & und./dist.	Huynh (1970)/Xi (1985)/PalDat (2023)
P. halleri (All.) Willd.	F	16, 32	3-colpate/3-colpate with 6-pantocolpate irregularly/3-colpate	L/L/—	—/dist./dist.	Huynh (1970) as *P. slavica* Reuss, *P. taurica* (Juz.) Aich. & Schwgl., *P. styriaca* (Pritz.) Simk. and *P. velezensis* (Beck) Aich. & Schwgl./Xi (1985) as *P. latifolia* Rupr./PalDat (2023)
P. rubra (Lamk.) Delarb.	F	16, 32	3-colpate/(3-colpate)	L/L	—/—	Huynh (1970) incl. var. *serotina* (Coste) Aich. & Schwgl. and ssp. *hispanica* Zimmerm. var. *nana* Aich. & Schwgl./Clarke *et al.* (1991)

Table 1 contd.

Taxa	Distr.[1)]	Chromosome number (2n)[2)]	Apertures	Grain size[3)]	Exine ornamentation[4)]	Palynological references
3-3. Sect. *Semicampanaria*	[A, B, C, D, E, F]					
3-3-1. Ser. *Pratenses*	[C, E, F]					
P. pratensis (L.) Mill.	E, F	16, 32	3-colpate/(3-colpate)/3-colpate/3-colpate	M-L/M/S/—	—/—/?ind./dist.	Huynh (1970) incl. var. *nigricans* (Storck) Aich. & Schwgl. and var. *flavescens* (Hazslinsky) Aich. & Schwgl./Clarke *et al.* (1991)/Xi (1985) as *P. nigricans* Störck/PalDat (2023)
P. montana (Hoppe) Zamels	F	16, 32, 48	3-colpate/3-colpate/3-colpate (with 30% anomalous grs.)	M-L/M/—	—/dist./dist.	Huynh (1970) incl. ssp. *balkana* (Velen.) Aich. & Schwgl. var. *australis* (Heuff.) Aich. & Schwgl./Xi (1985)/Nowicke & Skvarla (1995) as *P. aurstalis*
P. turczaninovii Krylov & Serg.	C	16	3-colpate/3-colpate	M-L/M	—/dist. & und.	Huynh (1970) incl. ssp. *eu-ambigua* (Zamels) Aich. & Schwgl. /Xi (1985)
3-3-2. Ser. *Semicampanaria*	[B, C]					
P. cernua (Thunb.) Bercht. & J.Presl.	B	16	3-colpate/3-colpate/3-colpate/3-colpate	M/M/S/S (SEM)	—/?ind. & und./ret.*/ind.	Huynh (1979)/Xi (1985)/Miyoshi *et al.* (2011), Fujiki *et al.* (2016)/Sarwar & Takahashi (2023)
P. chinensis (Bunge) Regel	C	16	3-colpate/3-colpate with 4-colpate and 6-pantocolpate	M/L	—/dist.	Huynh (1970)/Xi (1985)
P. dahurica (Fisch.) Spreng.	C	16	3-colpate/3-colpate with 6-pantocolpate	M/M	—/dist. & und.	Huynh (1970)/Xi (1985)
P. tongkangensis Y.N.Lee & T.C.Lee	B	16	—	—	—	—
3-3-3. Ser. *Albanae*	[A, C, D]					
P. albana Bercht. & J.Presl.	D	16	pantoporate/pantoporate (sometimes pores fused)	L /—	—/dist.	Huynh (1970)/Baladehi *et al.* (2013)
P. andina (Rupr.) Grossh.	D		—	—	—	—
P. georgica Rupr.	D	16	—	—	—	Huynh (1970) as *P. albana* ssp. *albana* var. *georgica* (Rupr.) Aich. & Schwgl.

Table 1 contd.

Taxa	Distr.1)	Chromosome number (2n)2)	Apertures	Grain size3)	Exine ornamentation4)	Palynological references
P. violacea Rupr.	D	16	—	—	—	Huynh (1970) as *P. albana* ssp. *albana* var. *violacea* (Rupr.) Aich. & Schwgl.
P. ambigua (Turcz. *ex* Pritz.) Juz.	D	16, 32	pantoporate/3-colpate	L/M	—/dist.	Huynh (1970) as *P. regeliana* (Maxim.) Aich. & Schwgl./Xi (1985) as *P. ambigua* Turcz. ex Pritz.
P. campanella (Regel & Tiling) Fisch. *ex* Krylov	A	16	pantoporate/8—12-pantoporate	M/M	—/? dist.	Huynh (1970)/Xi (1985)
P. wallichiana Ulbr.	A		Pantoporate	M	—	Huynh (1970)
P. tenuiloba (Hayek) Juz.	C	16	Pantoporate	M	—	Huynh (1970) as *P. bungeana* ssp. *tenuiloba* (Turcz.) Aich. & Schwgl.
P. sukaczewii Juz.	D		pantoporate/pantoporate together spiraperturate	M/M	—/dist. & und.	Huynh (1970) as *P. bungeana* ssp. *sukaczewii* (Juz.) Aich. & Schwgl./Xi (1985)
P. bungeana C.A.Mey. *ex* Ledeb.	C	16, 32	—	—	—	—
P. armena (Boiss.) Rupr.	D	16	Pantoporate	M	—	Huynh (1970) as *P. albana* ssp. *armena* (Boiss.) Aich. & Schwgl.
P. millefolia Ulbr.	A		pantoporate/pantoporate	M/M	—/? dist.	Huynh (1970)/Xi (1985)

1) Distr. (Geographical distribution following Sramkó *et al*. 2019): A, Central Asian mountains; B, Far East; C. Eastern Eurasia steppes; D, Caucasus Mts.; E, Western & Central Eurasia steppes; F, European grasslands; G, North America; H, European mountains.

2) According to Tamura (1995), Probatova *et al*. (2007), Nishikawa (2008), Grey-Wilson (2020), and < http://ccdb.tau.ac.il/home/>.

3) Average grain size in the longest axis: S, below 30 µm; M, 30 µm or more but below 40 µm; L, 40 µm or more.

4) Ornamentation: dist., microechinate with distinct tectal perforations; ind., microechinate without distinct tectal perforations; ret., reticulate; und., tectum undulate surface.

Most common palynological character states (3-colpate aperture, M in grain size, and dist. in exine sculpture) in *Pulsatilla* are underlined.

* Reticulate sculpture is probably due to the wrong sample (cf. Sarwar & Takahashi 2023).

material (perine, pollenkitt, and so on) and observe the surface ornamentation of the swollen pollen grains by SEM. The palynological trait of flat tectum versus undulate tectum (Nowicke and Skvarla, 1995) may be problematical for comparison at the species level. Palynological studies need to be performed on more species that have not yet been observed (Table 1). The pollen morphology of Far East Asian species (e.g., *P. magadanensis*, *P. tatewakii*, *P. sugawarai*, and *P. tongkangensis*) in particular should be clarified.

Palynological studies by LM should clarify aperture type and pollen grain size within a species or between populations. These palynological traits are related to chromosome number (cf. Table 1). The TEM study on *Pulsatilla* pollen wall has been conducted only in restricted species; *P. chinensis* and *P. campanella* in Xi (1985), and *P. grandis* in PalDat (2023). Structural diversity within the exine wall in *Pulsatilla* pollen is not yet known.

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