Pollen morphology of *Pieris* D. Don (Lyonieae, Ericaceae) and its taxonomic significance

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Pollen morphology of 6 species in the genus *Pieris* was examined using light and scanning electron microscopes. Judd's infrageneric classification of *Pieris* was reexamined in the light of new palynological characters. The genus *Pieris* is stenopalynous in having 3-colporate and oblate pollen tetrads. However, a continuous and serial variation in the exine sculpture, tetrad diameter, polar and equatorial length of pollen and apocolpial exine thickness was revealed within the genus. *Pieris nana* is characterized by having the smallest pollen and the smallest rugulae in the rugulate sculpture. Considering other distinct morphological characters, we confirm the recognition of monotypic subgenus *Arcterica* based on this species. Within the other subgenus, *Pieris*, members of the sections *Pieris* and *Phillyreoides* can be distinguished by subtle differences in colpus width, septum thickness and ratio of colpus length to tetrad diameter. *Pieris formosa* is distinguished by its psilate exine with clear secondary striate sculpture. *Pieris japonica* and *P. cubensis* are not well differentiated in the palynological characters, and act as a bridge between sect. *Pieris* and sect. *Phillyreoides*. On the basis of palynological characters a dichotomous key of these taxa was also prepared.

Key words: *Pieris*, pollen morphology, taxonomic significance.

The genus *Pieris* D. Don (Ericaceae–Vaccinioideae–Lyonieae) (Kron et al. 2002) comprises seven allopatric and rather distinctive species occurring in Eastern Asia, Eastern America, and Cuba (Judd 1982) and is closely related to several genera in the Andromedeae *sensu* Stevens (1971).

Until beginning of 20th century, the generic limit of *Pieris* is not very well defined. Some of its species such *as P. nana, P. floribunda* and *P. phillyreifolia* were segregated as distinct genera (Nuttall 1843, Small 1914, 1933, Makino 1961, Ohwi 1965). The genus *Pieris*, as circumscribed by Stevens (1971) along with the genus *Arcterica* Cov., is a member of *Lyonia* group of the tribe Andromedeae (Ericaceae subfamily Vaccinioideae). From the phenetic standpoint, Judd (1979) did not find sufficient morphological and anatomical distinctions between monotypic genus *Arcterica* and *Pieris* on the basis of 50 selected characters, and treated *Arcterica nana* as an isolated species within *Pieris*; *P. nana* (subgenus *Arcterica*). This genus is considered to be monophyletic group easily separated from related genera by a number of features; including the leaf arrangement and anatomy, timing of inflorescence development, flower, filament and spur morphology, seed morphology (Judd 1979, 1982), chromosome number, secondary metabolites (toxic diterpenes), and molecular characters (Kron et al. 1999, 2002). However, infrageneric classification of *Pieris* is sill in dispute. Judd (1982) has divided the genus *Pieris* into two subgenera; monotypic subgenus *Arcterica* (Cov.) Judd (including *P. nana*) and subgenus *Pieris*, which has been divided into two sections, sect. *Pieris* (including *P. japonica*, *P. formosa*, and *P. floribunda*) and sect. *Phillyreoides* Benth. & Hook. (including *P. phillyreifolia*, *P. cubensis*, and *P. swinhoei*). Recent combined phylogenetic analysis of phenotypic and molecular data shows that subgenus *Arcterica* composed of *P. nana* is sister to the other monophyletic subgenus *Pieris* including *P. formosa*, *P. floribunda* and *P. phillyreifolia* (Kron *et al.* 1999). But the monophyly of sect. *Pieris* is not supported in the combined analysis, thus subgenus *Pieris* needs further study.

Mainly light microscopic (LM) observations on pollen grains of *Pieris* have been carried out previously (Yang 1952, Ikuse 1956, 2001, Ueno 1962, Nair 1965, Huang 1972, Shimakura 1973, Nakamura 1980, Wang *et al.* 1995, Wei, 2003). Scanning electron microscopic (SEM) observation of pollen grains has been made for only a single species, *P. japonica* (Kurosawa 1991). Therefore, the present study examines the pollen morphology of the genus in detail with the help of both LM and SEM to discuss and review the present infrageneric classification.

Materials and Methods

The pollen of 6 species from the seven on so species in the genus *Pieris* was examined by using LM and SEM (Table 1). Polliniferous material used in this investigation was taken from dried herbarium specimens from the herbaria; KYO, S, SAPS and SAPT (the Botanic Garden, Hokkaido University, Sapporo).

Pollen was acetolysed following the technique of Erdtman (1960) modified by Takahashi (1987). For LM, the pollen was mounted in silicone oil (viscosity 3000 cs), and examined and measured with a Nikon Eclipse E200 microscope. The dimensions “D”, “P”, “E (d)” and “2f”, corresponding to the tetrad diameter, polar length, equatorial diameter and colpus length of pollen grain, were measured, and the D/d, P/E and 2f/D ratios were calculated (see Oldfield 1959). The measurements given in Table 2 are based on at least 10 grains from each specimen. Pollen size and shape classes follow Erdtman (1986). Pollen slides of all collection are deposited at the Hokkaido University Museum, Sapporo, Japan. For SEM, the acetolysed pollen samples were dehydrated in an ethanol series, and mounted and air dried on aluminum stubs from 70% ethanol, and sputter coated with Platinum-Palladium or Gold-Palladium by a HITACHI E102 ion sputter. Subsequently the prepared pollen was examined and photographed with a JEOL JSM-5310LV scanning electron microscope operated at 15 KV. Descriptive terminology follows Punt *et al* (1994).

Quantitative data of pollen morphological features (first ten characters shown in Table 2) were used for statistical calculations of total similarities between the pollen grains of six species with principal component analysis (PCA) by using the Excel Stat computer package.

Results

General pollen morphology of *Pieris*

Light microscopy observation: pollen grains united at tetrahedral tetrad (Fig. 1), rarely other configurations, viscin threads absent, size medium, or minute in *P. nana*, D 31.9 – 48.6 µm, P 17.1 – 24.3 µm, E 24.3 – 35.6 µm, P/E 0.66 – 0.72, oblate in shape, ratio of tetrad diameter to individual grain diameter (D/d) 1.28 – 1.37, 3-aperturate, apertures arranged according to "Fischer's Law", colporate, colpi distinct (Fig. 2), 13.8 – 29.0 µm long, width 0.6 – 2.0 µm, ratio of colpus length to tetrad diameter (2f/D) 0.33 – 0.60, wider at middle, somewhat obtuse to acute towards ends, colpus margin distinct, ora distinct, lalongate, 1.0 – 3.0 µm long, width 6.4 – 11.0 µm, costae present but not clear in *P. phillyreifolia* and *P. japonica*, exine tectate, apocolpial exine 1.9 – 2.6 µm thick, septum thickness 0.7 – 2.2 µm, apocolpial exine commonly thicker than septum except *P. formosa* with thicker septum. Exine sculpture commonly verrucate to finely rugulate or rugulate in *P. cubensis, P. phillyreifolia* and *P japonica*, or coarsely verrucate to coarsely rugulate in *P. formosa, P. koidzumiana* and *P. nana*.

SEM observations: apocolpial exine sculpture various from coarsely rugulate to psilate, the rugulae with secondary sculptures; faintly to finely and clearly striate (Figs. 3 – 10). Exine sculpture along the colpi similar to that appearing at distal pole, but mesocolpial exine having a tendency to decrease in lateral extension of the rugulae with more distinct units, colpus membrane granular to granuloid.

In principal component analysis (PCA) using the LM characters, dots from each species occupy each distinct range, so we can generally compare the palynological features of six *Pieris* species in this analysis (Fig. 11).

Pollen characteristics of *Pieris*

Subgenus *Arcterica* (monotypic, species examined: *P. nana*)

Pollen grains few in number at both LM slide and SEM stub. Pollen surface uneven and rugged, exine sculpture coarsely rugulate, the rugulae faintly (Fig. 3) to finely striate (Fig. 4). In PCA, *P. nana* is situated at the lower left edge of total variation of the *Pieris* pollen (Fig. 11).

Subgenus *Pieris*

Section *Pieris* (species examined: *P. japonica, P. koidzumiana* and *P. formosa*)

Colpus membrane coarsely granulate in *P. japonica*. Pollen surface uneven to somewhat flat and rugged, exine sculpture coarsely rugulate, the rugulae faintly to finely striate in *P. japonica* and *P. koidzumiana* (Figs. 5 – 7) to psilate with clearly striate sculpture in *P. formosa* (Fig. 8). In PCA, members of this section are situated at the middle of total variation, along with *P. cubensis* of Sect. *Phillyreifolia*. Among the species *P. formosa* shows the highest value in the second component (Fig. 11).

Section *Phillyreoides* (species examined: *P. phillyreifolia* and *P. cubensis*)

Most grains somewhat shrink in *P. phillyreifolia*. Pollen surface uneven and rugged, exine sculpture coarsely rugulate(-psilate), the rugulae loosely arranged, and finely and clearly granulate-striate in *P. phillyreifolia* (Fig. 9) or striate in *P. cubensis* (Fig. 10). In PCA, *P. phillyreifolia* is situated at the right edge of total variation of the *Pieris* pollen which shows the highest value of the first component and *P. cubensis* pollen could not be separated from *P. japonica* pollen (Fig. 11).

Discussion

All species of *Pieris* examined in this study have 3-colporate and oblate pollen tetrads having similar rugulate to rugulate-psilate (rarely psilate) exine with secondary striate sculpture, which suggests that the genus including *P. nana* is, as a whole a group of closely related entity. However, SEM observations show a variation in exine sculpture within the genus (Figs. 3 – 10). There is a more or less continuous and serial variation in the exine sculpture from coarsely rugulate through coarsely rugulate-psilate to psilate, among the *Pieris* species.

Pieris nana has the smallest pollen tetrad (D = 31.9μm) and the smallest rugulae in size (compare Figs. 3 – 4 and 5 – 10). This species has also very distinctive morphological characters viz. low shrub with small, entire and usually whorled leaves, roughened-papillose filament, and anthers with poorly developed disintegration tissue etc., differing from the other species of the genus (Judd 1982). The fewer number of pollen grains in both LM slide and SEM stub might be due to the presence of poorly developed disintegration tissue in anthers or all specimens might be collected at very late flowering stage. The distinctiveness of the *P. nana* pollen is also supported by PCA (lower left edge of total variation) using 10 palynological characters based on LM observations (Fig. 11).The PCA also indicate that among the remaining five species, *P. japonica, P. koidzumiana*, *P. formosa* and *P. cubensis* are relatively closer together and situated at the middle of total variation, and *P. phillyreifolia* also has a distinct (most right edge) position (Fig. 11). This results of PCA support the division of the genus *Pieris* into two subgenera; monotypic subgenus *Arcterica* (including *P. nana*) and the other subgenus *Pieris* composed of the remaining species. The infrageneric classification of *Pieris* proposed by Judd (1982) is generally supported by the palynological characters, with some exceptions viz. *P. cubensis* of sect. *Phillyreoides* shows a closer relationship with the members of sect. *Pieris* (especially *P. japonica*) (Fig. 11).

Within the subgenus *Pieris*, P/E ratio shows a difference between two sections (Table 2). Section *Pieris* possesses relatively larger value (0.72) of P/E, and sect. *Phillyreoides* possesses the smaller value (0.68), except for *P. japonica* of sect. *Pieris* that has the P/E value 0.66, even lower than sect. *Phillyreoides*. Colpus length, width and ratio of colpus length to tetrad diameter (2f/D) show distinct differences among the species (Table 2). Pollen grains of the sect. *Pieris* possess comparatively smaller but wider colpus (13.8 – 20.2 μm and 1.6 – 2.0 μm, respectively) compared to those of the sect. *Phillyreoides* (18.6 – 29.0 μm and 0.6 – 0.7 μm, respectively). The 2f/D value is relatively smaller (0.33 – 0.47) in the sect. *Pieris*, and larger (0.47 – 0.60) in the sect. *Phillyreoides*. Distinct differences in P/E and 2f/D ratios were also reported previously among the sections of the genus *Enkianthus* (Sarwar & Takahashi 2005) and different subfamilies of Ericaceae (Warner & Chinappa 1986). These two palynological characters might be of special taxonomic importance in Ericaceae. Distinct differences have also been found in apocolpial exine and septum thickness between the two sections (Table 2): sect. *Pieris* have comparatively thicker apocolpial exine and septum (2.1 – 2.6 µm and 1.1 – 2.2 µm, respectively) than those of the sect. *Phillyreoides* (1.9 – 2.3 µm and 0.7 – 1.0 µm, respectively).

Judd (1982) considered *P. japonica* as a variable species; it includes a number of synonyms at the species level, e.g. *P. popowi* Palibin, *P. taiwanensis* Hayata, *P.polita* W.W. Sm. & Jeffrey, *P. koidzumiana* Ohwi. *Pieris koidzumiana* occupy the intermediate space between *P. japonica* and *P. formosa* in PCA (Fig. 11). But in exine sculpture *P. koidzumiana* (Fig. 7) is more similar to *P. japonica* (Figs. 5 – 6) than *P. formosa* (Fig. 8). Thus palynological characters do not positively support the separation of *P. koidzumiana* from *P. japonica*. Judd (1982) also did not confirm the species status of *P. koidzumiana*, because he did not find any clear gap between *P. koidzumiana* and *P. japonica* in leaf shape or marginal dentation, although he recognized some distinct morphological differences between them. However, considering other distinction in the morphological characters of *P. koidzumiana*: e.g. the leaves oblanceolate with more blunt tip, rachis densely puberulous, flowers longer, etc., compared to those of *P. japonica* (Yamazaki 1993), it is better to separate *P. koidzumiana* from *P. japonica* as a distinct species, endemic to Ryukyu Islands, Japan.

Neither the close relationships between *P. japonica* and *P. formosa*, nor *P. phillyreifolia* with *P. cubensis* and *P. japonica*, which were found in morphological and anatomical characters (Judd 1982), is supported by our palynological observations (Figs. 5 – 11). However, *P. japonica* pollen can not be separated from that of *P. cubensis* in PCA (Fig. 11), and the exine sculpture is also similar between the two species (Figs. 5 – 6 and 10). *Pieris japonica* and *P. cubensis* act as a bridge between the two sections *Pieris* and *Phillyreoides*. A close relationship between *P. formosa* and *P. phillyreifolia* suggested by Kron et al. (1999) was not supported by PCA (Fig. 11), but the distinctness of secondary striate sculpture is their common palynological feature (Figs. 8 – 9). Under SEM, *P. formosa* is readily distinguished by its psilate exine with clear secondary striate sculpture (Fig. 8) among *Pieris* species. Other species have a serial variation in the coarsely rugulate-psilate exine sculpture (rugulae faintly to finely striate). Further research including the study of more specimens and combined analysis of morphological and molecular data from other regions is needed to clarify the relationships among the *Pieris* species.

Key to the species of *Pieris* based on pollen morphology

1a. Pollen grains minute, pollen surface uneven and rugged, exine sculpture coarsely rugulate, the rugulae smallest -- *P. nana*

1b. Pollen grains mediae, pollen surface uneven and rugged to somewhat flat, exine sculpture coarsely rugulate to psilate, the rugulae relatively larger -- 2

2a. 2f/D 0.33 – 0.47, colpus width 1.6 – 2.0 µm, septum relatively thicker (1.1 – 2.2 µm) ------- 3

3a. D/d 1.29 – 1.32, 2f/D 0.43 – 0.47, distal exine thicker than septum, septum thickness 1.1 – 1.7 µm, pollen surface uneven and rugged, exine sculpture coarsely rugulate, the rugulae faintly to finely striate -- 4

4a. D/d 1.29, P/E 0.66, 2f/D 0.47, septum thickness 1.1µm ------------ *P. japonica*

4b. D/d 1.32, P/E 0.72, 2f/D 0.43, septum thickness 1.7µm ------- *P. koidzumiana*

3b. D/d 1.36, 2f/D 0.33, septum thicker than distal exine, septum thickness 2.2µm pollen surface somewhat flat, exine sculpture psilate with clearly secondary striate -- *P. formosa*

2b. 2f/D 0.47 – 0.60, colpus width 0.6 – 0.7 µm, septum relatively thinner (0.7 – 1.0 µm) ------- 5

5a. D/d 1.37, 2f/D 0.60, septum thickness 1.0µm, the rugulae finely and clearly granulate-striate -- *P. phillyreifolia*

5b. D/d 1.28, 2f/D 0.47, septum thickness 0.7µm, the rugulae finely and clearly striate --- *P. cubensis*

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Table 1. Specimens sampled for the palynological studies of *Pieris*, LM Light microscope, SEM Scanning electron microscope. Infrageneric classification follows Judd (1982)

Taxon	Collector and No.	Voucher Information
Subgenus ***Arcterica***		
P. nana (Maxim.) Makino	Fukuda 180, 181 (LM, SEM)	Japan: Honshu, Rikutyu, Mt. Hayatine, 19 June 1932 (KYO)
	Takahashi et al. 2571 (SEM)	Japan: Hokkaido, Prov. Kitami, Monbetsu-gun, Shirataki-mura, Mt. Taira-yama, alt. 1770m, 29 June 1982 (SAPS)
Subgenus ***Pieris***		
Section ***Pieris***		
P. japonica (Thunb.) D. Don	Takahashi 457 (LM, SEM)	Japan: Tokyo, Koishikawa Botanical Garden, cultivated, petals pink, 23 March 1980 (SAPS)
	Sasao s.n. (SEM)	Taiwan: Prov. Taichû, Kantojum, 20 November 1931 (SAPS)
P. koidzumiana Ohwi	Sonohara 10 (LM, SEM)	Japan: Okinawa Islands, Kunigami, Hendona, no date (KYO)
P. formosa (Wallich) D. Don	McLaren 32F (LM, SEM)	China: Yunnan, Pai-ching, 13 April 1936 (Ex Herb. Hort. Bot. Reg. Edin.) (KYO)
Section ***Phillyreoides***		
P. phillyreifolia (Hook.) DC.	Newcombe 267 (LM, SEM)	USA: Georgia, Charlton Co., Okefenokee National Wildlife Refuge, 13 September 1982 (SAPT)
P. cubensis (Griseb.) Small	Ekman (Pl. Ind. Occ. 16387) (LM, SEM)	Cuba: Prov. Pinar del Rio, Sierra de los Organos, 31 March 1923 (S)

Table 2. Variation in pollen characters of *Pieris* showing value in μm and standard deviation, D tetrad diameter, P polar axis, E equatorial axis, minimum – maximum values in parenthesis. Sculpture by SEM: CR Coarsely rugulate, CRP Coarsely rugulate-psilate, P Psilate, * Secondary striate sculpture distinct. Parentheses mean indistinctness

Name of Subgenus/ Section/Species	D	P	E(d)	D/d	P/E	Colpus		2f/D	Apocolpial exine thickness	Septum thickness	Sculpture by SEM
						Length (2f)	Width				
Subgenus ***Arcterica***											
P. nana	31.9±1.4	17.1±1.9	24. 3±2.5	1.31	0.70	17.3±2.8	2.0±0.3	0.54	2.2±0.2	1.6±0.3	CR* - CR(P) $^{(*)\,-}$ *
	(30.0-34.6)	(14.9-20.6)	(21.1-30.0)	(1.15-1.42)	(0.64-0.78)	(13.9-21.6)	(1.7-2.4)	(0.45-0.63)	(1.9-2.4)	(1.2-1.9)	
Subgenus ***Pieris***											
Section ***Pieris***											
P. japonica	42.8±2.4	22.0±1.5	33.1±1.4	1.29	0.66	20.2±2.5	1.6±0.6	0.47	2.4±0.3	1.1±0.3	CR - CR(P) $^{(*)}$
	(39.3-46.2)	(19.5-24.4)	(31.4-36.0)	(1.14-1.41)	(0.60-0.74)	(16.5-24.8)	(0.7-2.5)	(0.36-0.58)	(2.1-3.0)	(0.7-1.5)	
P. koidzumiana	39.3±2.1	21.4±1.6	29.7±1.5	1.32	0.72	16.9±2.8	1.6±0.5	0.43	2.6±0.3	1.7±0.2	CR - CRP $^{(*)}$
	(37.4-42.7)	(19.0-23.5)	(27.1-31.2)	(1.23-1.40)	(0.65-0.78)	(13.4-20.6)	(0.7-1.9)	(0.36-0.54)	(2.2-2.9)	(1.4-2.2)	
P. formosa	41.5±2.6	22.1±1.4	30.5±2.5	1.36	0.72	13.8±1.2	2.0±0.3	0.33	2.1±0.2	2.2±0.2	P*
	(37.2-45.6)	(19.9-27.7)	(27.8-34.8)	(1.28-1.45)	(0.65-0.81)	(12.5-16.8)	(1.4-2.4)	(0.27-0.45)	(1.9-2.4)	(1.9-2.4)	
Section ***Phillyreoides***											
P. phillyreifolia	48.6±1.6	24.3±2.0	35.6±2.2	1.37	0.68	29.0±2.8	0.7±0.3	0.60	1.9±0.5	1.0±0.4	CRP*
	(47.0-51.2)	(20.8-27.7)	(32.2-39.6)	(1.26-1.49)	(0.62-0.74)	(24.8-33.0)	(0.5-1.5)	(0.53-0.64)	(1.3-2.8)	(0.5-1.7)	
Pieris cubensis	39.3±1.2	20.8±1.0	30.7±1.2	1.28	0.68	18.6±1.4	0.6±0.1	0.47	2.3±0.3	0.7±0.2	CRP*
	(38.0-41.3)	(19.8-22.8)	(29.4-33.0)	(1.24-1.33)	(0.63-0.72)	(17.3-21.4)	(0.5-0.8)	(0.42-0.52)	(2.0-2.8)	(0.5-1.2)	

Table 3. Cumulative variance and eigen vectors of principal component analysis (PCA) using ten palynological characters based on LM for 6 species of *Pieris*. Character abbreviations corresponding to Table 2

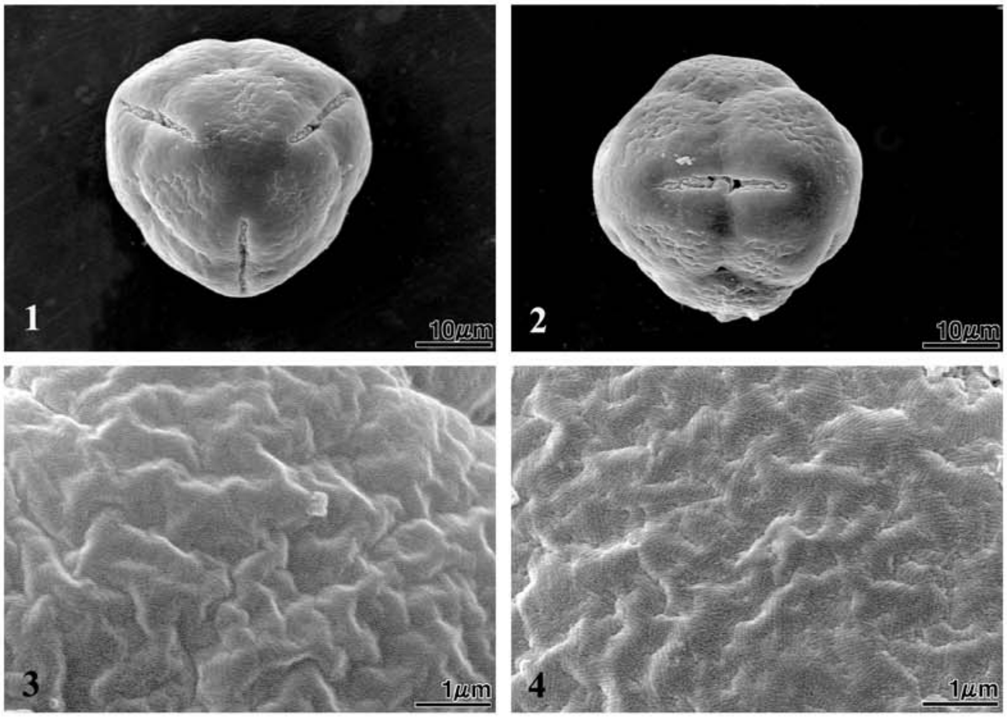
Principal Component Axis	1	2	3
Cumulative Variance (%)	39.926	60.622	76.138
Characters		Eigen vectors	
D	0.417	0.336	-0.030
P	0.355	0.437	-0.149
E	0.432	0.186	-0.297
D/d	-0.024	0.337	0.579
P/E	-0.155	0.460	0.230
Colpus length (2f)	0.416	-0.118	0.364
Colpus width	-0.363	0.067	0.110
2f/D	0.228	-0.378	0.465
Apocolpial exine thickness	-0.185	0.032	-0.353
Septum thickness	-0.312	0.416	0.126

Legend:

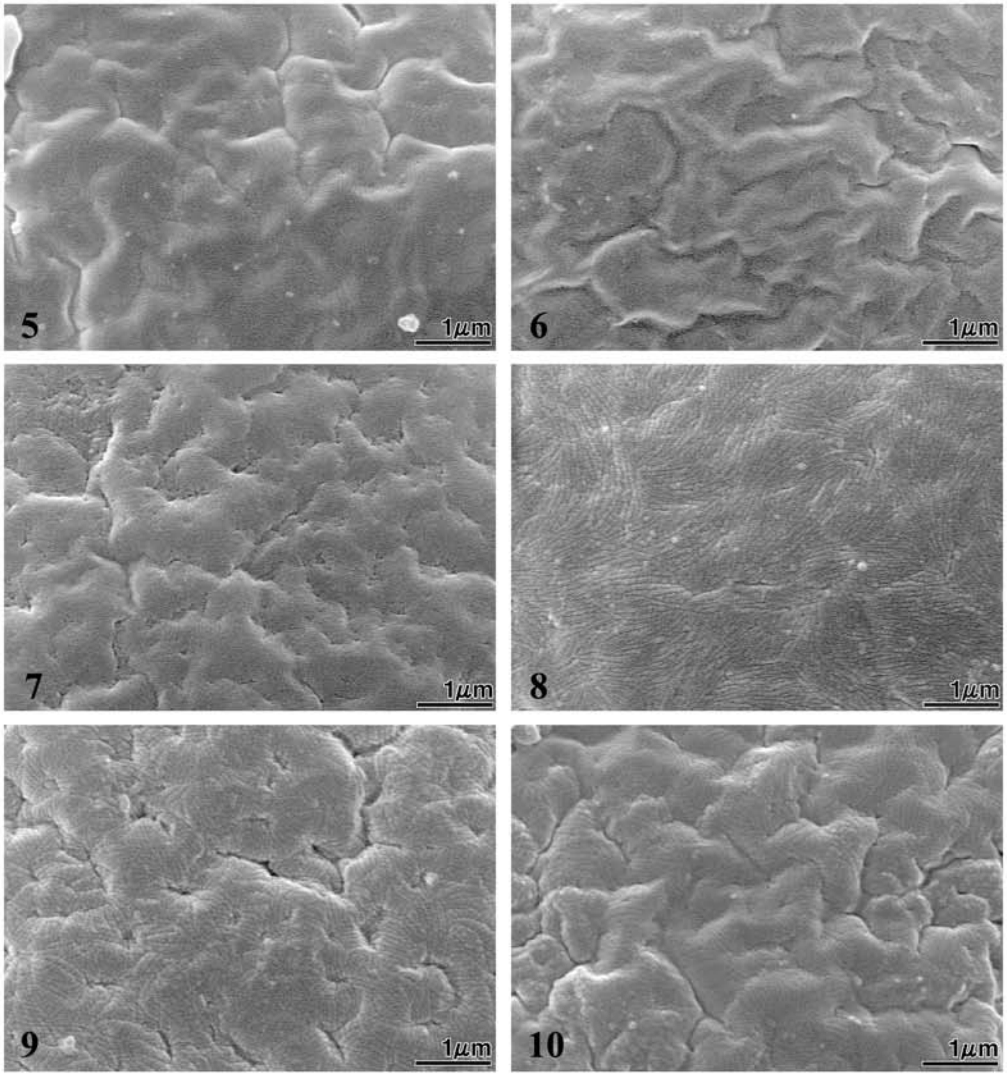
Figs. 1 – 4. SEM micrographs of pollen grains. 1: Pollen tetrad at polar view (*P. formosa*). 2: Pollen tetrad at equatorial view showing aperture (*P. formosa*). 3 – 4: Apocolpial exine sculpture of *P. nana* (Takahashi et al. 2571 and Fukuda 180, respectively).

Figs. 5 – 10. SEM micrographs of apocolpial exine sculpture. 5 – 6: *P. japonica* (Takahashi 457 and Sasao, s.n., respectively). 7: *P. koidzumiana*. 8: *P. formosa*. 9: *P. phillyreifolia*. 10: *P. cubensis*.

Fig. 11. Two dimensional diagram of component 1 and 2 of pollen grains of six *Pieris* species based on principal component analysis using ten palynological characters.



Figs. 1-4.



Figs. 5-10.

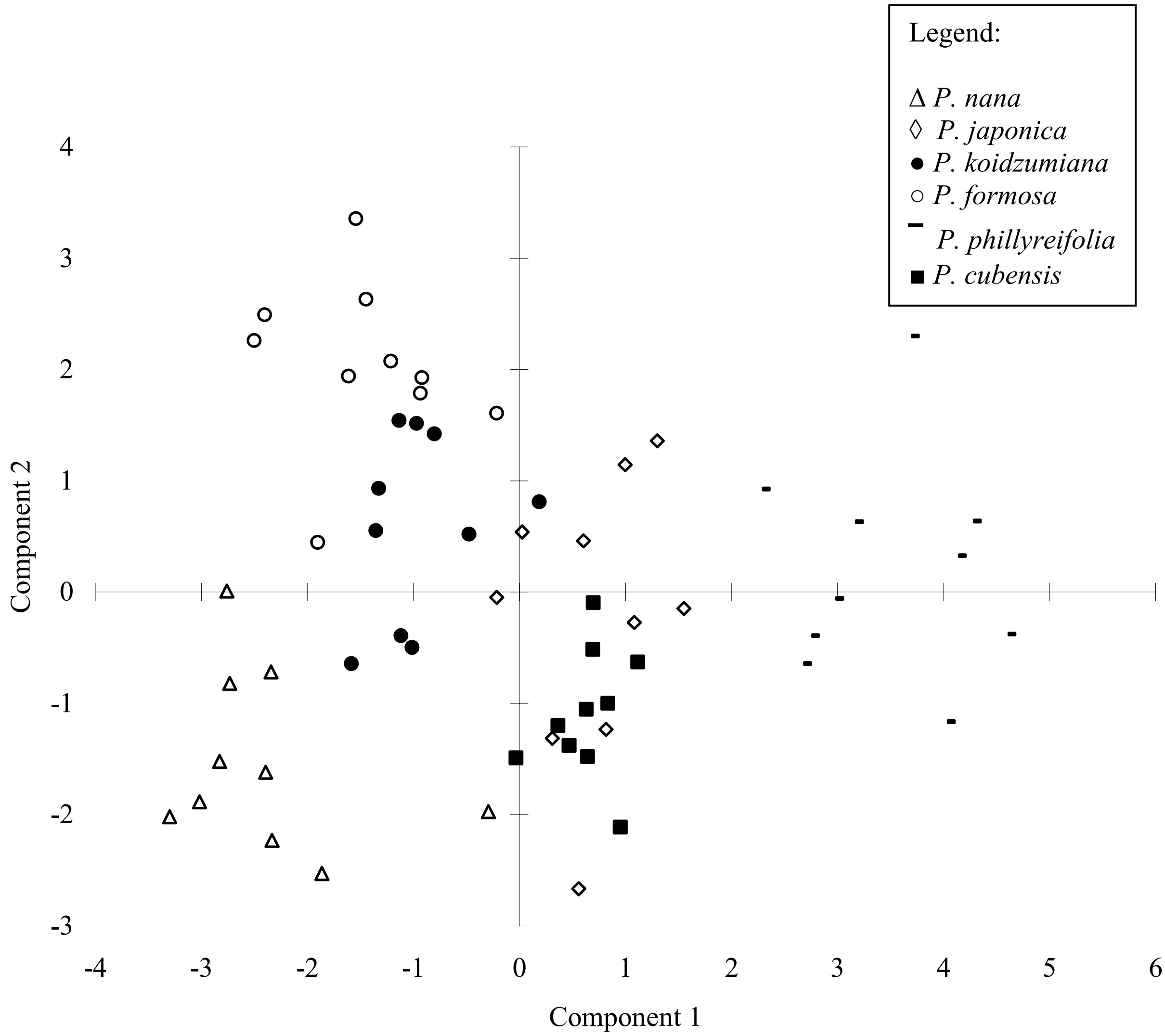

Legend:
Δ P. nana
◊ P. japonica
● P. koidzumiana
○ P. formosa
– P. phillyreifolia
■ P. cubensis
Component 2
Component 1
4
3
2
1
0
-1
-2
-3
-4
-3
-2
-1
0
1
2
3
4
5
6

Fig. 11