

Phylogenetic evaluation of pollen and orbicule morphology in Rosaceae tribe Neillieae (subfamily Amygdaloideae)

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Palynological characteristics were examined in Rosaceae tribe Neillieae, comprising the genera *Neillia*, *Physocarpus* and *Stephanandra*. *Physocarpus insularis* and some taxa of *Spiraea* were also examined to evaluate the potential usefulness of pollen traits in taxonomic and phylogenetic studies of Rosaceae. Pollen grains of Neillieae are monads, tricolporate, small to medium in size [polar axis (*P*) = 13.4–45.8 µm, equatorial diameter (*E*) = 14.3–39.9 µm] and oblate to prolate in shape (*P/E* = 0.68–1.70). Sexine ornamentation in *Neillia* and *Stephanandra* is perforate, whereas *Physocarpus* and *Spiraea* have striate pollen. Spherical orbicules with a central perforation (donut-shaped) were consistently found in Neillieae, but these were absent in *P. insularis* and all taxa of *Spiraea*. Our palynological results provide strong evidence for the merging of *Neillia* and *Stephanandra*, and pollen morphological data supported the recent phylogenetic hypothesis that *P. insularis* should be a member of *Spiraea*.

ADDITIONAL KEYWORDS: *Neillia* – *Physocarpus insularis* – pollen morphology – *Spiraea* – *Stephanandra*.

INTRODUCTION

Rosaceae tribe Neillieae Maxim, a small group of *c.* 25 taxa in subfamily Amygdaloideae, have traditionally been considered to contain three genera, *Neillia* D. Don, *Physocarpus* (Cambess.) Raf. and *Stephanandra* Siebold & Zucc. (Maximowicz, 1879; Schulze-Menz, 1964; Kalkman, 2004). Members of the tribe are characterized by lobed and palmatinerved leaves, with persistent or deciduous stipules, relatively small follicles and ovoid shiny seeds with abundant endosperm (Vidal, 1963; Kalkman, 2004). Among the members of this tribe, *Neillia*, *Stephanandra* and *Physocarpus amurensis* (Maxim.) Maxim. are restricted to East Asia; *P. alternans* (M.E. Jones) J.T. Howell, *P. capitatus* (Pursh) Kuntze, *P. malvaceus* (Greene) Kuntze and *P. monogynus* (Torr.) J.M. Coulter are native to western North America; and *P. opulifolius* (L.) Maxim. is only found in eastern North America (Oh, 2002, 2015, 2016).

Although morphological and phylogenetic studies consistently indicated that Neillieae are a monophyletic tribe in Rosaceae (Morgan, Soltis & Robertson, 1994; Potter *et al.*, 2002), relationships within the tribe have been contradictory. For example, Benthams & Hooker (1865), Greene (1889) and Jones (1893) treated *Physocarpus* as part of *Neillia* and treated *Stephanandra* as a separate genus, whereas Kuntze (1891) classified all members of Neillieae in *Physocarpus* (i.e. with *Neillia* and *Stephanandra* as synonyms of *Physocarpus*), all characterized by having abundant endosperm. Although many taxonomists recognize three genera (*Neillia*, *Stephanandra* and *Physocarpus*) in Neillieae (Rehder, 1940; Schulze-Menz, 1964; Robertson, 1974; Takhtajan, 1997; Kalkman, 2004), recent molecular phylogenetic results suggested *Stephanandra* should be included in *Neillia* (Oh & Potter, 2003, 2005; Oh, 2006, 2016). Nevertheless, only a few morphological synapomorphies are found in *Neillia* including follicle and seed characters (Oh, 2006).

Taxonomic inconsistencies have also been found in *P. insularis* (Nakai) Nakai, an endemic Korean species restricted to Ulleung Island (Nakai, 1918; Oh, 1978;

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Lee & Yang, 1981). Kim, Kim & Sun (2000) suggested *P. insularis* was a synonym of *Spiraea chamaedryfolia* L. var. *ulmifolia* (Scop.) J. DuRoi, after comparing leaf shape, carpel connation, follicle dehiscence and distribution of stellate hairs in these taxa. Recently, a comprehensive systematic study of *P. insularis*, based on morphological and DNA sequence analyses, suggested this species should be included in *Spiraea* L. (Oh *et al.*, 2010); it could be distinguished from *S. chamaedryfolia* by larger leaf blades, subcordate or truncate leaf bases and a higher number of stamens (Oh *et al.*, 2010). Consequently, this taxon was transferred from *Physocarpus* to *Spiraea* as *S. insularis* (Nakai) H. Shin, Y. D. Kim & S. H. Oh (Shin, Kim & Oh, 2011). However, no clear morphological characters distinguishing *S. insularis* from *S. chamaedryfolia* have been described so far.

Recently, detailed pollen characters have been used to support phylogenetic hypotheses (Welsh, Stefanović & Costea, 2010; Chen & Xia, 2011; Hong & Pan, 2012; Ma, Bramley & Zhang, 2016). In particular, pollen morphology in Rosaceae has provided valuable diagnostic characters at the genus (Eide, 1981; Ueda & Tomita, 1989; Hebda & Chinnappa, 1990b, 1994; Lee, Jung & Lee, 1993) and species (Eide, 1981; Hebda & Chinnappa, 1990a; Ueda, 1992; Wrońska-Pilarek & Jagodziński, 2011) levels and has been used to resolve phylogenetic relationships (Chung, Elisens & Skvarla, 2010; Lee *et al.*, 2011; Wrońska-Pilarek & Jagodziński, 2011; Shi, Wen & Lutz, 2013; Wrońska-Pilarek, Bocianowski & Jagodziński, 2013). However, palynological studies on Neillieae conducted so far were restricted to a few taxa with a narrow geographical distribution and were based on light microscopy (LM) (Naruhashi & Toyoshima, 1979; Chang, 1986; Hebda, Chinnappa & Smith, 1991; Lee *et al.*, 1993; Wang *et al.*, 1997; Zhou, Wei & Wu, 1999). Thus, detailed palynological studies might help to clarify the generic delimitation in Neillieae. In addition, the morphological details of orbicules, small sporopollenin particles produced by the secretory tapetum, may also provide valuable phylogenetic information (Huysmans *et al.*, 1997, 2010; Huysmans, El-Ghazaly & Smets, 2000; Huysmans & Smets, 1998; Geeraerts *et al.*, 2009; Verstraete *et al.*, 2011, 2014). Although studies on orbicules are scarce in Rosaceae, recent descriptions of the orbicules in tribe Sorbarieae (subfamily Amygdaloideae) were of systematic importance (Song, Moon & Hong, 2016).

Therefore, the present study aims to (1) describe and illustrate pollen morphology and the presence of orbicules in members of Neillieae; (2) elucidate the systematic relationships among all taxa in Neillieae by comparing pollen morphological characteristics, based on the consensus phylogenetic tree of Oh &

Potter (2005); and (3) re-evaluate the systematic position of *P. insularis* (= *S. insularis*) using palynological evidence.

MATERIAL AND METHODS

PLANT MATERIAL

All currently accepted taxa of Neillieae (*Neillia*: nine species, five varieties; *Physocarpus*: seven species; *Stephanandra*: three species, one variety) and three *Spiraea* taxa, to clarify the taxonomic position of *P. insularis*, were studied using 41 herbarium specimens. Anthers were collected from plants deposited in the herbaria A, GH, KB, KH, KHUS and WU. These acronyms follow those of Thiers (2016) [continuously updated]. In addition, some plants were collected from natural populations in Korea. Details of voucher specimens are summarized in the appendix. To confirm the consistency of morphological characteristics, we compared at least two specimens from each taxon, when possible.

MICROSCOPIC OBSERVATION

Pollen grains were prepared using the critical point drying method (CPD; Moon *et al.*, 2008). Because CPD allows the presence of orbicules to be observed, we used CPD for scanning electron microscopy (SEM) observations. Although the standard acetolysis method (ACE; Erdtman, 1960) can distort pollen grain shape, resulting in striking size differences from CPD pollen grains (Moon *et al.*, 2008), pollen grains were also analysed using ACE to compare their size and shape with previously published data, which are largely based on ACE. Pollen grains subjected to ACE were observed under LM (BX41 Laboratory Microscope, Olympus, Melville, USA), and their size was measured using a digital camera for microscopes (MDX-30, Shinwoo Optics, Anyang, Korea). Measurements were based on at least 30 pollen grains. For SEM observations, dried stamens were rehydrated with Agepon® (Agfa Gevaert, Leverkusen, Germany; Agepon wetting agent: distilled water, 1:200). Fresh and rehydrated samples were dehydrated in an acetone dehydration series (Moon *et al.*, 2008) and then immersed in carbon dioxide before CPD (using an SPI-13200J-AB instrument, SPI Supplies, West Chester, USA). Dried anthers were fixed to aluminium stubs with double adhesive carbon tape, and the locules of the stamens were carefully opened using a cactus needle. Stubs were coated with gold using an ion-sputtering device (E-1045, Hitachi, Tokyo, Japan), and pollen grains and orbicules were observed using a field emission SEM (FE-SEM, S-4700, Hitachi, Tokyo, Japan) at an accelerating voltage of 10 kV with a working distance of 10–12 mm.

Table 1. Overview of major pollen characters of all taxa studied within Neillieae and related species

Taxa	T	P (μm)	E (μm)	Shape				CL (μm)				ET (μm)	Type
				O	SO	OS	S	PS	SP	P			
<i>Neillia affinis</i> var. <i>affinis</i>	A	29.7–32.9–38.5	23.9–32.4–38.0	-	+	++	-	++	+	+	24.2–28.1–34.3	1.13–1.44–2.14	I
	C	18.9–21.0–24.2	21.7–23.9–26.9	-	+	++	-	-	-	-	14.1–18.5–21.9	/	I
<i>Neillia affinis</i> var. <i>longisepala</i>	A	27.8–31.2–36.2	25.1–26.8–36.2	-	-	-	-	++	+	+	21.0–24.8–29.2	1.27–1.58–1.91	I
	C	18.3–20.4–21.9	19.8–22.2–25.7	-	+	++	-	+	-	-	15.6–16.6–18.2	/	I
<i>Neillia affinis</i> var. <i>pauciflora</i>	A	25.9–30.5–33.1	30.6–33.7–36.9	-	+	++	-	-	-	-	22.4–25.8–28.1	1.16–1.56–1.76	I
	C	17.8–19.9–21.7	20.2–22.9–24.7	-	++	++	-	-	-	-	14.1–16.3–18.2	/	I
<i>Neillia affinis</i> var. <i>polygyna</i>	A	29.6–31.1–32.1	32.3–34.0–36.8	-	+	++	-	-	-	-	24.2–25.7–27.6	1.13–1.68–2.12	I
	C	17.9–20.2–23.9	20.6–23.4–25.9	-	++	++	-	-	-	-	13.5–17.7–21.2	/	I
<i>Neillia gracilis</i>	A	23.1–27.1–31.6	23.8–28.3–32.2	-	+	++	-	+	+	-	19.6–22.5–26.1	1.07–1.36–1.62	I
	C	16.7–19.2–22.1	18.5–20.7–24.6	-	+	+	-	+	-	-	15.4–17.8–20.5	/	I
<i>Neillia rubiflora</i>	A	26.8–32.6–39.6	20.3–28.0–31.7	-	-	+	-	++	++	-	21.8–26.1–30.8	0.96–1.60–2.43	I
	C	22.0–22.7–24.0	23.6–24.6–26.3	-	+	+	-	-	-	-	18.3–19.5–21.0	/	I
<i>Neillia serratisepala</i>	A	25.6–27.6–33.7	28.2–29.6–32.1	-	++	++	+	+	-	-	19.8–22.8–27.3	1.16–1.59–2.14	I
	C	18.5–22.3–25.5	22.2–23.4–26.2	-	+	+	+	+	-	-	17.2–20.4–22.9	/	I
<i>Neillia sinensis</i> var. <i>sinensis</i>	A	22.5–33.7–44.3	20.7–30.1–38.1	-	-	+	+	+	++	-	17.7–28.4–36.5	1.15–1.65–2.55	I
	C	17.8–22.0–23.9	20.3–24.7–28.1	+	++	++	-	+	-	-	15.6–19.4–21.9	/	I
<i>Neillia sinensis</i> var. <i>hypomalaca</i>	A	31.2–35.7–38.8	28.1–32.0–35.3	-	-	+	-	+	++	-	26.0–28.6–31.8	1.24–1.72–2.08	I
	C	17.7–21.0–23.1	21.7–23.7–28.1	-	++	++	-	-	-	-	14.3–18.4–21.3	/	I
<i>Neillia sparsiflora</i>	A	28.7–31.8–36.0	24.2–26.9–29.5	-	-	-	-	++	++	+	19.8–22.8–27.3	1.16–1.59–2.14	I
	A	26.6–32.5–41.0	21.6–29.1–35.2	-	+	+	+	++	++	+	21.1–28.2–35.8	1.15–1.60–2.37	I
<i>Neillia thibetica</i> var. <i>thibetica</i>	C	18.7–20.8–22.5	18.5–20.5–25.8	-	+	+	-	++	-	-	15.6–17.1–18.5	/	I
	A	25.7–32.7–41.9	22.3–28.5–36.5	-	+	+	-	+	+	++	18.6–26.8–35.1	0.96–1.46–2.01	I
<i>Neillia thibetica</i> var. <i>lobata</i>	C	18.1–19.3–21.4	19.5–22.3–27.1	-	++	++	-	-	-	-	15.2–17.3–19.1	/	I
	A	20.3–24.6–29.1	19.1–22.5–25.5	-	-	+	-	++	++	-	15.8–19.7–23.1	1.07–1.48–2.12	I
<i>Neillia thyrsiflora</i>	C	13.4–17.2–19.8	17.3–19.8–22.9	+	++	+	+	-	-	-	11.9–15.1–17.0	/	I
	A	28.6–35.1–39.6	29.0–35.0–39.9	+	+	++	-	++	+	-	22.4–28.8–34.7	1.31–1.98–2.44	I
<i>Neillia uekii</i>	C	15.4–18.2–20.0	17.6–21.6–24.5	-	++	+	-	-	-	-	12.5–15.7–17.7	/	I
	A	27.4–31.2–37.8	18.7–23.0–30.3	-	-	-	-	-	++	++	21.8–24.7–30.4	1.21–1.56–2.08	II-1
<i>Physocarpus alternans</i>	C	14.4–15.8–16.7	15.4–17.4–18.8	-	+	++	-	-	-	-	12.2–13.9–15.3	/	II-1
	A	25.5–30.5–38.2	19.5–26.0–34.6	-	-	+	-	+	++	+	21.5–26.4–34.7	0.92–1.27–1.80	II-1
<i>Physocarpus amurensis</i>	C	15.5–18.6–24.9	18.2–21.3–26.2	-	+	++	-	+	-	-	12.4–16.5–24.0	/	II-1
	A	26.4–31.5–35.9	23.9–27.9–32.5	-	-	+	-	++	++	-	22.3–26.1–30.6	1.01–1.43–1.92	II-1
<i>Physocarpus capitatus</i>	C	22.8–26.1–27.8	22.3–24.7–27.7	-	-	+	+	++	+	-	20.7–23.1–25.4	/	II-1
	A	17.3–19.4–22.0	15.2–17.0–19.0	-	-	+	-	++	++	-	13.0–15.5–18.7	1.13–1.36–1.62	II-2
<i>Physocarpus insularis</i>	C	10.9–11.9–12.8	11.6–12.7–14.5	-	-	+	+	+	-	-	9.60–10.4–11.6	/	II-2
	A	30.4–40.7–45.8	26.0–31.7–37.6	-	-	-	-	+	++	+	23.1–33.5–37.5	1.91–2.37–2.92	II-1
<i>Physocarpus malvaceus</i>	C	19.1–21.8–23.1	19.8–20.9–21.7	-	-	+	-	++	-	-	15.8–18.9–21.1	/	II-1

Table 1. Continued

Taxa	T	P (µm)	E (µm)	Shape		OS	S	PS	SP	CL (µm)		ET (µm)	Type
				O	SO								
<i>Physocarpus monogynus</i>	A	28.1–33.6–42.3	21.5–27.9–31.7	-	-	-	-	++	++	21.6–28.1–36.0	1.86–2.01–2.71		II-1
	C	16.5–20.4–24.3	18.1–21.6–25.1	-	++	++	-	+	-	14.8–18.3–22.7	/		II-1
<i>Physocarpus opulifolius</i>	A	25.2–32.4–40.4	20.1–28.4–33.0	-	-	+	-	+	++	19.8–26.6–32.9	1.13–1.98–3.43		II-1
	C	19.9–24.4–27.3	20.2–24.1–27.4	-	-	++	+	++	+	17.5–21.7–25.2	/		II-1
<i>Spiraea chamaedryfolia</i>	A	20.5–22.1–23.0	17.0–18.9–19.5	-	-	-	-	+	++	16.0–17.5–18.0	1.08–1.12–1.51		II-2
	C	10.1–11.3–12.1	11.3–12.7–13.6	-	-	++	+	+	-	8.86–9.86–11.1	/		II-2
<i>Spiraea pseudocrenata</i>	A	17.0–17.6–18.0	14.0–15.3–16.5	-	-	-	-	++	+	13.0–13.9–15.0	0.85–0.96–1.11		II-2
	C	11.6–12.1–12.9	10.4–11.4–11.8	-	-	+	-	++	+	10.0–10.8–11.8	/		II-2
<i>Spiraea pubescens</i>	A	17.5–18.2–19.0	15.0–15.4–16.0	-	-	-	-	++	++	13.0–14.4–15.0	1.13–1.19–1.27		II-2
	C	11.9–12.9–13.8	11.3–13.2–14.6	-	-	++	+	++	-	10.2–11.8–12.6	/		II-2
<i>Stephanandra chinensis</i>	A	24.9–26.1–27.5	25.2–27.0–29.8	-	-	++	+	+	-	19.7–21.4–23.7	1.24–1.48–1.78		I
	C	14.1–15.9–18.6	14.3–16.8–18.3	-	+	++	-	++	-	11.7–13.9–15.5	/		I
<i>Stephanandra incisa</i> var. <i>incisa</i>	A	21.2–25.8–34.2	18.4–25.5–31.9	-	+	++	+	++	+	14.8–21.3–27.4	1.12–1.46–2.23		I
	C	14.5–17.4–22.0	15.5–18.6–22.2	-	+	++	+	+	-	12.3–15.6–20.1	/		I
<i>Stephanandra incisa</i> var. <i>quadrifissa</i>	A	22.0–24.2–26.9	20.3–24.2–27.4	-	-	++	+	+	+	17.0–19.8–22.8	0.97–1.41–1.82		I
	C	14.2–16.6–18.5	16.0–18.6–20.9	-	+	++	-	-	-	12.4–14.2–16.4	/		I
<i>Stephanandra tanakae</i>	A	24.1–27.6–30.5	18.5–23.0–29.0	-	-	+	+	++	+	18.9–23.8–26.9	1.12–1.76–2.32		I
	C	17.1–19.6–24.8	17.8–20.4–22.5	-	+	++	+	++	-	12.9–17.4–23.3	/		I

Numbers refer to minimum–mean–maximum. T, treatment method; A, acetolysis; C, critical point dried; P, polar axis; E, equatorial diameter; O, oblate; SO, sub-oblate; OS, oblate-spheroidal; S, spherical; PS, prolate-spheroidal; SP, sub-prolate; P, prolate; CL, colpus length; ET, exine thickness; -, absent; +, present; ++, dominance; /, no data.

Table 2. Measurements of equatorial diameter in polar view (EDPV), apocolpus side (AS), Polar Area Indices (PAI) of the pollen grains, width of ridge (WR), width of valley (WV), diameter of perforation (PR) of sexine ornamentations ($n = 15$) of taxa in the tribe Neillieae and related species. -, absent

Taxa	EDPV (μm)	AS (μm)	PAI	WR (μm)	WV (μm)	PR (μm)
<i>Neillia affinis</i> var. <i>affinis</i>	21.1–(23.9)–27.4	6.28–(7.98)–9.85	0.34 (0.27–0.41)	-	-	0.08–(0.15)–0.25
<i>Neillia affinis</i> var. <i>longisepala</i>	20.9–(22.9)–25.7	6.96–(8.24)–9.56	0.36 (0.31–0.44)	-	-	0.08–(0.14)–0.19
<i>Neillia affinis</i> var. <i>pauciflora</i>	20.2–(23.4)–25.8	7.10–(9.09)–10.8	0.39 (0.32–0.47)	-	-	0.08–(0.15)–0.30
<i>Neillia affinis</i> var. <i>polygyna</i>	20.8–(23.6)–25.7	7.43–(8.90)–10.3	0.38 (0.31–0.44)	-	-	0.12–(0.15)–0.22
<i>Neillia gracilis</i>	19.1–(22.2)–24.8	4.80–(5.68)–6.45	0.26 (0.22–0.29)	-	-	0.10–(0.15)–0.20
<i>Neillia rubiflora</i>	22.5–(23.7)–25.0	7.47–(8.31)–9.20	0.35 (0.31–0.39)	-	-	0.11–(0.19)–0.33
<i>Neillia serratisepala</i>	21.0–(23.7)–27.3	6.96–(7.85)–8.95	0.33 (0.27–0.43)	-	-	0.10–(0.17)–0.22
<i>Neillia sinensis</i> var. <i>sinensis</i>	21.7–(24.4)–27.6	5.77–(8.10)–11.3	0.33 (0.25–0.47)	-	-	0.09–(0.18)–0.31
<i>Neillia sinensis</i> var. <i>hypomalaca</i>	21.7–(24.4)–26.7	6.70–(8.40)–12.6	0.34 (0.27–0.52)	-	-	0.10–(0.18)–0.38
<i>Neillia thibetica</i> var. <i>thibetica</i>	22.3–(25.8)–30.2	7.17–(8.31)–9.69	0.32 (0.28–0.37)	-	-	0.12–(0.20)–0.29
<i>Neillia thibetica</i> var. <i>lobata</i>	21.0–(23.2)–25.7	4.07–(6.32)–8.17	0.27 (0.18–0.33)	-	-	0.09–(0.15)–0.20
<i>Neillia thyrsiflora</i>	17.6–(19.5)–21.3	5.05–(6.23)–8.36	0.32 (0.27–0.42)	-	-	0.09–(0.12)–0.17
<i>Neillia uekii</i>	19.2–(22.0)–25.2	4.92–(6.90)–9.04	0.31 (0.25–0.39)	-	-	0.10–(0.23)–0.39
<i>Physocarpus alternans</i>	16.6–(18.0)–19.9	3.34–(4.16)–5.13	0.23 (0.20–0.26)	0.15–(0.20)–0.25	0.07–(0.12)–0.14	0.07–(0.12)–0.15
<i>Physocarpus amurensis</i>	17.8–(21.2)–24.2	2.48–(4.93)–6.70	0.24 (0.11–0.32)	0.16–(0.24)–0.32	0.10–(0.17)–0.27	0.10–(0.16)–0.25
<i>Physocarpus capitatus</i>	23.5–(24.9)–28.8	4.93–(6.24)–9.82	0.25 (0.20–0.34)	0.20–(0.28)–0.32	0.07–(0.13)–0.19	0.06–(0.11)–0.19
<i>Physocarpus insularis</i>	12.0–(13.7)–15.7	2.87–(3.46)–4.92	0.25 (0.20–0.32)	0.10–(0.11)–0.14	0.14–(0.17)–0.20	0.03–(0.07)–0.12
<i>Physocarpus malvaceus</i>	18.2–(20.3)–23.1	4.44–(4.89)–5.22	0.24 (0.22–0.26)	0.13–(0.22)–0.32	0.16–(0.23)–0.34	0.11–(0.19)–0.33
<i>Physocarpus monogynus</i>	19.4–(21.8)–26.1	4.45–(5.29)–6.75	0.25 (0.20–0.32)	0.20–(0.32)–0.45	0.08–(0.15)–0.24	0.12–(0.15)–0.21
<i>Physocarpus opulifolius</i>	20.9–(25.2)–28.3	3.64–(5.37)–7.17	0.21 (0.15–0.26)	0.27–(0.36)–0.52	0.12–(0.22)–0.37	0.11–(0.20)–0.30
<i>Spiraea chamaedryfolia</i>	10.6–(12.7)–13.6	2.44–(3.19)–3.89	0.25 (0.21–0.30)	0.16–(0.18)–0.20	0.05–(0.07)–0.11	0.04–(0.06)–0.10
<i>Spiraea pseudocrenata</i>	10.2–(11.4)–12.0	2.74–(3.16)–3.71	0.28 (0.23–0.34)	0.11–(0.12)–0.14	0.02–(0.07)–0.12	0.03–(0.05)–0.07
<i>Spiraea pubescens</i>	10.9–(11.8)–12.9	2.46–(3.02)–3.94	0.26 (0.21–0.31)	0.15–(0.18)–0.21	0.02–(0.05)–0.12	0.02–(0.04)–0.08
<i>Stephanandra chinensis</i>	14.9–(17.6)–21.8	4.16–(5.58)–8.10	0.32 (0.23–0.47)	-	-	0.10–(0.19)–0.30
<i>Stephanandra incisa</i> var. <i>incisa</i>	15.8–(18.5)–21.6	3.31–(5.42)–8.14	0.29 (0.21–0.41)	-	-	0.08–(0.17)–0.43
<i>Stephanandra incisa</i> var. <i>quadrifissa</i>	17.7–(19.3)–22.5	4.87–(5.84)–6.87	0.30 (0.26–0.36)	-	-	0.09–(0.15)–0.36
<i>Stephanandra tanakae</i>	17.9–(20.0)–21.9	5.06–(5.88)–7.50	0.29 (0.25–0.37)	-	-	0.10–(0.17)–0.31

Twelve variables were determined based on SEM micrographs and measured using Macnification software (version 2.0, Orbicule Inc., Leuven, Belgium): P (polar axis); E (equatorial diameter); P/E; CL (colpus length); CL/P, EDPV (equatorial diameter in polar view); AS (apocolpus side); PAI (polar area index); WR (width of ridge); WV (width of valley); PR (diameter of perforation) (Tables 1–2); and OB (orbicule diameter) (Table 3).

To determine if pollen characteristics allowed taxa to be grouped, a principal component analysis (PCA) was performed in PC-ORD Version 5.31 (McCune & Mefford, 2011) and the result was presented in a two-dimensional plot of the first and second principal components. Pollen sexine ornamentation types were mapped onto the phylogenetic tree of Oh & Potter (2005) to evaluate their phylogenetic significance. Pollen terminology followed Punt *et al.* (2007) and Verstraete *et al.* (2011).

RESULTS

SIZE AND SHAPE

Pollen grains were monads and their size varied from small to medium ($P = 10.9\text{--}45.8\text{ }\mu\text{m}$, $E = 11.6\text{--}39.9\text{ }\mu\text{m}$; Table 1). *Physocarpus capitatus* had the largest pollen grains (CPD: $P = 26.1 \pm 1.94\text{ }\mu\text{m}$, $E = 24.7 \pm 1.85\text{ }\mu\text{m}$), and *Stephanandra chinensis* Hance (CPD: $P = 15.9 \pm 1.43\text{ }\mu\text{m}$, $E = 16.8 \pm 1.32\text{ }\mu\text{m}$) and *P. insularis* (= *Spiraea insularis*) (CPD: $P = 11.9 \pm 0.59\text{ }\mu\text{m}$, $E = 12.7 \pm 0.72\text{ }\mu\text{m}$) had the smallest pollen grains (Table 1). Polar length was strongly correlated with equatorial diameter ($r = 0.829$, $P < 0.001$). Within a taxon, pollen grains examined using ACE were always smaller than those examined using CPD (Table 1). Size variation was generally nonsignificant at the generic level. The outline of the pollen grains was mostly triangular in polar view, although obtuse, straight or convex patterns were also observed (Figs 1A–F, 2A–C, G, I). In equatorial view,

Table 3. Occurrence and morphology of orbicules in the tribe Neillieae related species ($n = 30$)

Taxa	Density	Diameter	Shape	Ornamentation	Association
<i>Neillia affinis</i> var. <i>affinis</i>	va	0.58–(0.75)–0.94	do	gr	emb
<i>Neillia affinis</i> var. <i>longisepala</i>	va	0.54–(0.64)–0.81	do	gr	emb
<i>Neillia affinis</i> var. <i>pauciflora</i>	va	0.54–(0.80)–1.08	do	gr	emb
<i>Neillia affinis</i> var. <i>polygyna</i>	va	0.53–(0.64)–0.86	do	gr	emb
<i>Neillia gracilis</i>	va	0.71–(0.89)–1.17	do	ps	emb
<i>Neillia rubiflora</i>	a	0.59–(0.76)–1.03	do	ps	emb
<i>Neillia serratisepala</i>	a	0.59–(0.73)–0.90	do	ps	emb
<i>Neillia sinensis</i> var. <i>sinensis</i>	a	0.49–(0.71)–1.09	do	ps	emb
<i>Neillia sinensis</i> var. <i>hypomalaca</i>	a	0.67–(0.82)–0.97	do	ps	emb
<i>Neillia sparsiflora</i>	a	0.62–(0.74)–1.01	do	ps	emb
<i>Neillia thibetica</i> var. <i>thibetica</i>	a	0.63–(0.91)–1.16	do	gr	agg, emb
<i>Neillia thibetica</i> var. <i>lobata</i>	a	0.82–(1.06)–1.24	do	ps	agg, emb
<i>Neillia thyrsiflora</i>	a	0.49–(0.59)–0.69	do	gr	emb
<i>Neillia uekii</i>	a	0.66–(0.85)–1.11	do	ps	agg, emb
<i>Physocarpus alternans</i>	va	0.51–(0.60)–0.70	do	ps	agg, emb
<i>Physocarpus amurensis</i>	va	0.47–(0.61)–0.85	do	ps	agg, emb
<i>Physocarpus capitatus</i>	va	0.55–(0.72)–0.91	do	ps	agg, emb
<i>Physocarpus insularis</i>	-	-	-	-	-
<i>Physocarpus malvaceus</i>	va	0.62–(0.75)–0.89	do	gr	agg, emb
<i>Physocarpus monogynus</i>	va	0.52–(0.73)–1.01	do	ps	agg, emb
<i>Physocarpus opulifolius</i>	va	0.44–(0.58)–0.77	do	ps	agg, emb
<i>Spiraea chamaedryfolia</i>	-	-	-	-	-
<i>Spiraea pseudocrenata</i>	-	-	-	-	-
<i>Spiraea pubescens</i>	-	-	-	-	-
<i>Stephanandra chinensis</i>	a	0.34–(0.55)–0.73	do	ps	agg, emb
<i>Stephanandra incisa</i> var. <i>incisa</i>	a	0.32–(0.50)–0.90	do	ps	emb
<i>Stephanandra incisa</i> var. <i>quadrifissa</i>	va	0.39–(0.50)–0.61	do	ps	agg, emb
<i>Stephanandra tanakae</i>	a	0.55–(0.83)–1.12	do	gr	emb

Density: a, abundant (orbicules cover a large part of the locule wall); va, very abundant (locule surface almost invisible due to the orbicules, cf. Verstraete *et al.*, 2011). Diameter: minimum–(mean)–maximum. Shape: do, doughnut-shaped. Ornamentation: gr, granulate; ps, psilate. Association: agg, aggregated; emb, embedded. -, absent.

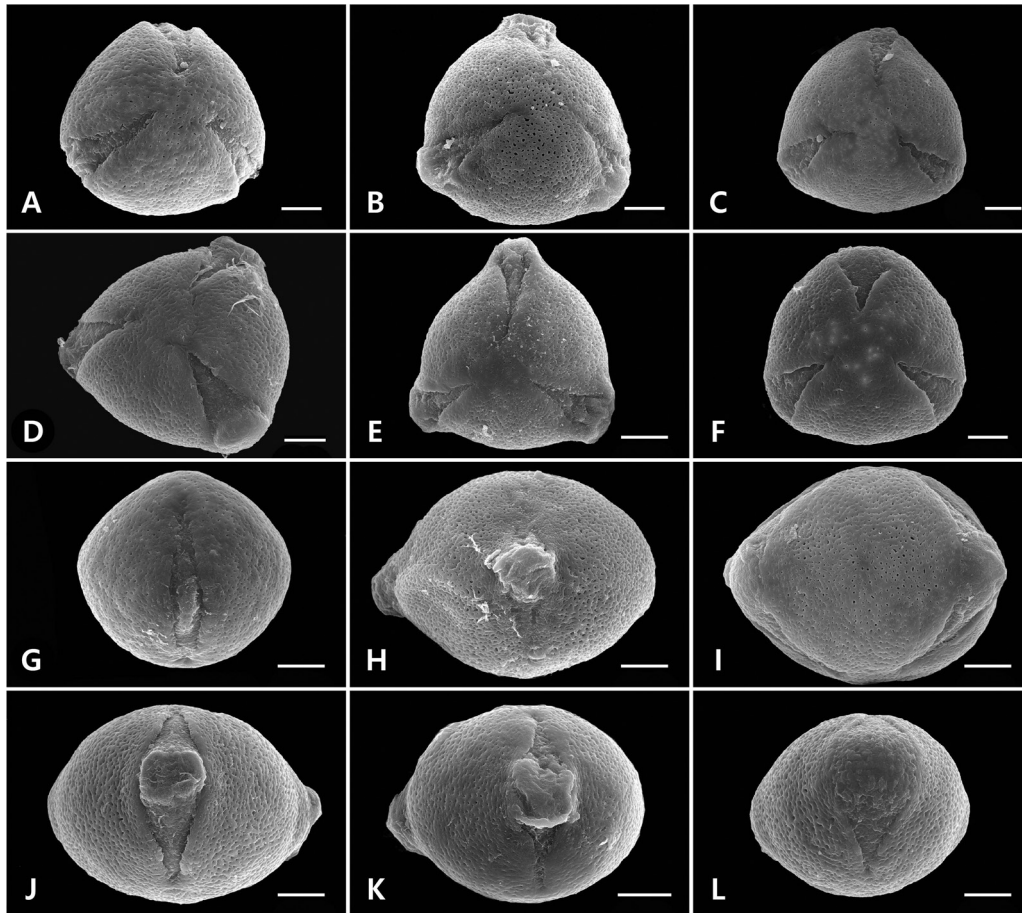


Figure 1. SEM micrographs of pollen grains of *Neillia*. (A–F), The outline variation in polar view of pollen grains. (G–L), Variation of pollen shape in the equatorial view. (A, G), *N. affinis* var. *affinis*. (B, H), *N. sinensis* var. *sinensis*. (C, I), *N. sinensis* var. *hypomalaca*. (D, J), *N. thibetica* var. *lobata*. (E, K), *N. thyrsoflora*. (F, L), *N. uekii*. All pollen grains on figures are critical-point dried (CPD) materials. All scale bars: 5 µm.

pollen grain shapes ranged from oblate to prolate ($P/E = 0.68–1.70$; Figs 1G–L, 2D–F, J, L).

APERTURES

All taxa had tricolporate pollen grains (Figs 1–2). Simple colpi were symmetrically distributed, elongated and narrowed toward the poles, with granular aperture membranes (Figs 1–2). Colpus length ranged from 9.60 µm [*Physocarpus insularis* (= *Spiraea insularis*)] to 37.5 µm (*P. malvaceus*) and was strongly correlated with P ($r = 0.971$, $P < 0.001$) and E ($r = 0.819$, $P < 0.001$). Colpus ends were mostly acute and pointed, and the membrane was covered with irregularly shaped granules. Polar area index varied from 0.11 to 0.52 (Table 2).

SEXINE ORNAMENTATION

Two distinct types (I, II) of sexine ornamentation were observed. In Type II, two subtypes were recognized

based on striae patterns and on the diameter of the perforations in the valley (Fig. 3).

Type I: Perforate – *Neillia* and *Stephanandra* (Fig. 3A–F, L)

Sexine ornamentation was perforate, with circular to subcircular perforations in the tectum but no supra-tectal sculpturing elements (striae). Type I ornamentation was found in: *Neillia affinis* Hemsl. var. *affinis* (Fig. 3A), *N. affinis* var. *longisepala* Cullen, *N. affinis* var. *pauciflora* (Rehder) J.E.Vidal (Fig. 3B), *N. affinis* var. *polygyna* Cardot ex J.E.Vidal, *N. gracilis* Franch., *N. rubiflora* D.Don, *N. serratisepala* H.L.Li, *N. sinensis* Oliv. in Hook. var. *sinensis* (Fig. 3C), *N. sinensis* var. *hypomalaca* (Rehder) Hand.-Mazz. (Fig. 3D), *N. sparsiflora* Rehder, *N. thibetica* Bureau & Franch. var. *thibetica* (Fig. 3E), *N. thibetica* var. *lobata* (Rehder) T.T.Yu, *N. thyrsoflora* D.Don, *N. uekii* Nakai (Fig. 3F), *Stephanandra chinensis*, *S. incisa* (Thunb.) Zabel

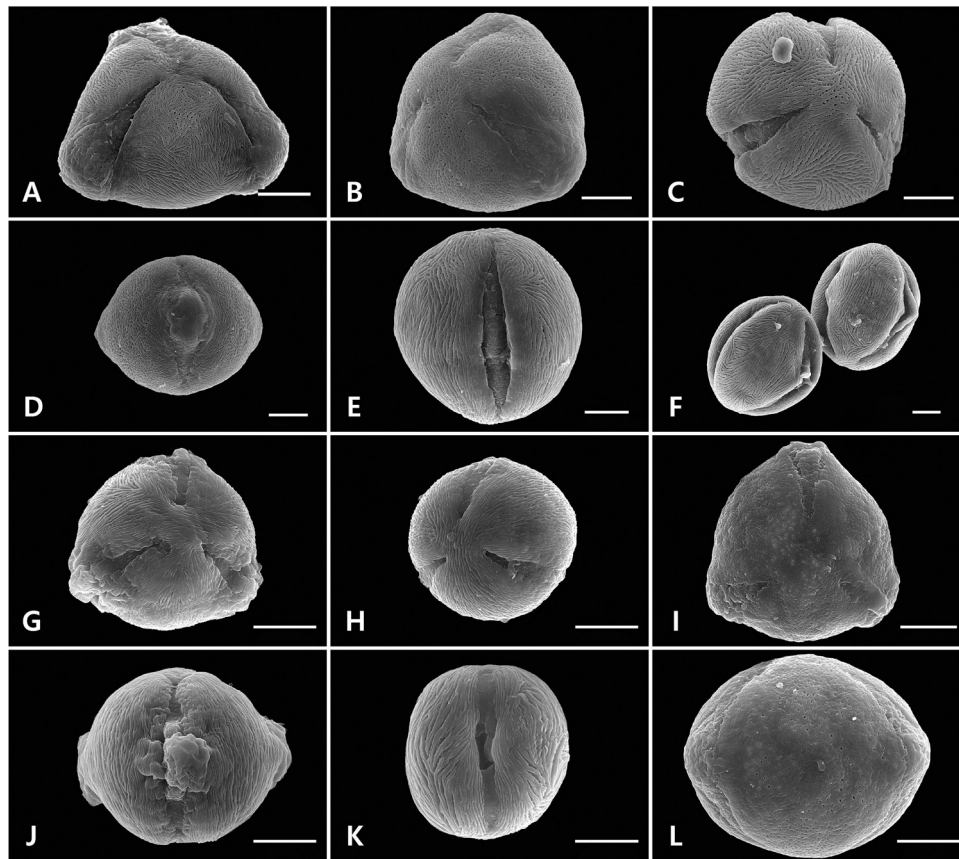


Figure 2. SEM micrographs of pollen grains of *Physocarpus*, *Stephanandra* and related species. (A–C, G–I), The outline variation in polar view of pollen grains. (D–F, J–L), Variation of pollen shape in the equatorial view. (A), *P. alternans*. (B, D), *P. amurensis*. (C, E), *P. malvaceus*. (F), *P. opulifolius*. (G, J), *P. insularis*. (H), *Spiraea chamaedryfolia*. (I, L), *Stephanandra incisa* var. *incisa*. (K), *Spiraea pubescens*. All pollen grains on figures are critical-point dried (CPD) materials. All scale bars: 5 μm .

var. *incisa* (Fig. 3L), *S. incisa* var. *quadrifissa* (Nakai) T.B.Lee and *S. tanakae* (Franch. & Sav.) Franch. & Sav.

Type II: Striate – *Physocarpus* and *Spiraea* (Fig. 3G–K)

Sexine ornamentation was striate with perforations. This type presented supratectal ridges separated by valleys with tectal perforations and could be divided into two subtypes.

Subtype II-1: Striate with macroporations (0.1–0.4 μm)

Ridges were short to medium in length and the extension before a change in direction was usually < 3 μm . Ridges showed some looping and crossing at the intercolpium region. Taxa with subtype II-1 ornamentation were: *Physocarpus alternans*, *P. amurensis*, *P. capitatus*, *P. malvaceus* (Fig. 3G), *P. monogynus* (Fig. 3H) and *P. opulifolius* (Fig. 3I).

Subtype II-2: Striate with microporations (0.03–0.09 μm)

Ridges were long and with few anastomoses. They extended from one pole to the other, parallel to the colpus (Fig. 3J, K). Taxa with subtype II-2 ornamentation were: *Physocarpus insularis* (Fig. 3J), *Spiraea chamaedryfolia* (Fig. 3K), *S. pseudocrenata* Nakai and *S. pubescens* Turcz.

ORBICULE MORPHOLOGY

Orbicules were found in all studied taxa of Neillieae (Fig. 4), except in *P. insularis* (Fig. 4J). Orbicules were also absent in all *Spiraea* spp. studied, including *S. chamaedryfolia* (Fig. 4K), which is related to *P. insularis*. Density, size, shape and ornamentation of orbicules and their association with the tapetal membrane varied among taxa (Table 3), but they were all spherical with a central perforation (doughnut-shaped, Fig. 4). According to their distribution patterns, orbicules were classified into ‘very abundant’ or ‘abundant’. Neillieae orbicules had an average diameter of $0.69 \pm 0.16 \mu\text{m}$,

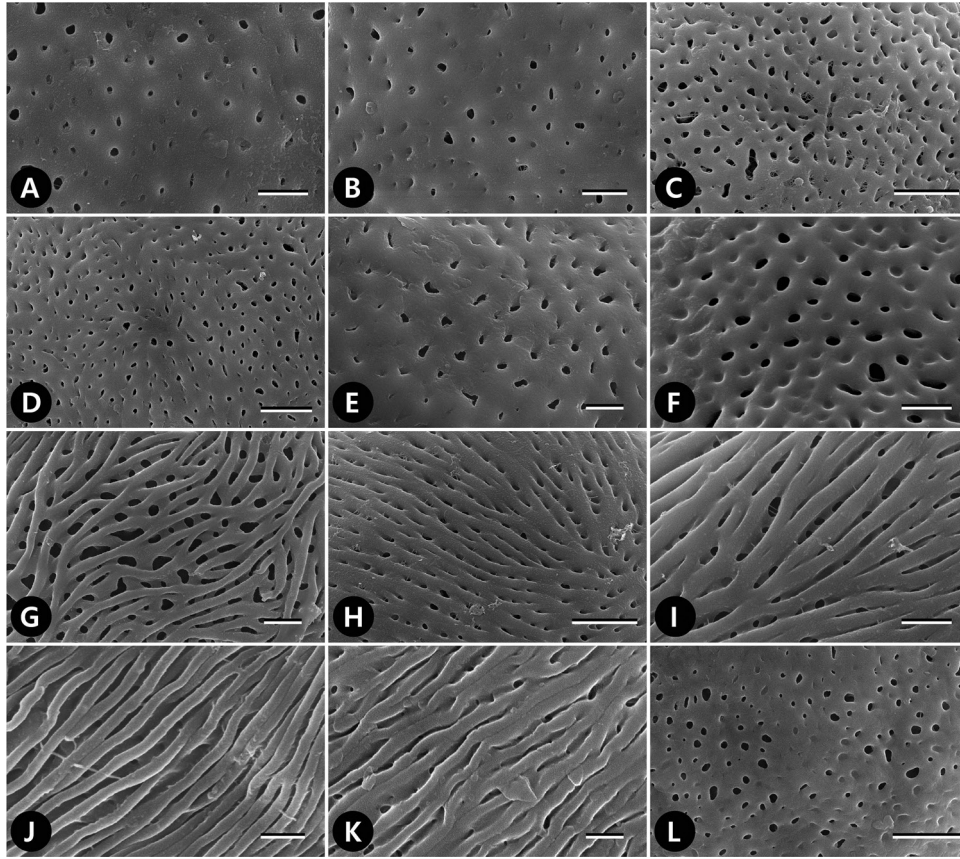


Figure 3. SEM micrographs of variation of sexine ornamentation in tribe Neillieae. (A–F, L), Variations of perforate sexine ornamentation (type I). (G–I), Variations of striate-macroporification (type II-1). (J, K), Variations of striate-microporification (type II-2). (A), *Neillia affinis* var. *affinis*. (B), *N. affinis* var. *pauciflora*. (C), *N. sinensis* var. *sinensis*. (D), *N. sinensis* var. *hypomalaca*. (E), *N. tibetica* var. *tibetica*. (F), *N. uekii*. (G), *Physocarpus malvaceus*. (H), *P. monogynus*. (I), *P. opulifolius*. (J), *P. insularis*. (K), *Spiraea chamaedryfolia*. (L), *Stephanandra incisa* var. *incisa*. All pollen grains on figures are critical-point dried (CPD) materials. Scale bars: C, D, H, L, 2.0 μ m; A, B, E, F, G, I, 1.0 μ m; J, K, 0.5 μ m.

the largest being found in *N. tibetica* var. *lobata* ($1.06 \pm 0.10 \mu$ m) and the smallest in *S. incisa* var. *incisa* ($0.50 \pm 0.11 \mu$ m) (Table 3). Orbicule diameter was significantly correlated with P ($r = 0.214$, $P < 0.001$) and E ($r = 0.265$, $P < 0.001$). The surface ornamentation of the orbicule was defined as psilate (Fig. 4C, F, G–I, L) or granulate (Fig. 4D, E). Occasionally, orbicules were aggregated (Fig. 4G–I). In all studied taxa, orbicules were partly or entirely fused with the inner locule wall, particularly at the tapetal membrane (Fig. 4).

PRINCIPAL COMPONENT ANALYSIS (PCA)

The first two principal components accounted for 77.61% of the variation (Table 4). The first principal component (PC 1) explained 52.36% of the variation and was strongly associated with pollen grain size (P, E, EDPV and AS), PR and OB and the second principal component (PC 2) explained 25.25% of the variation and was strongly associated with variability of sexine ornamentation (WR,

WV), CL and PAI (Table 4). The PCA biplot revealed clusters of operational taxonomic units (OTUs) corresponding to sexine ornamentation. Moreover, pollen morphology clearly separated taxa into three groups (Fig. 5). The first group consisted of OTUs from all taxa of *Neillia* and *Stephanandra*. The second group, which was distributed on the negative side of PC 2, included *Physocarpus* spp., except *P. insularis*. The third group grouped *P. insularis* with three species of *Spiraea* (Fig. 5). These four taxa shared the same palynological characteristics such as subtype II-2 sexine ornamentation (i.e. striate with microporifications) and the absence of orbicules.

DISCUSSION

POLLEN MORPHOLOGICAL VARIATION IN NEILLIEAE

All previous palynological studies on Neillieae were conducted based on pollen examined using ACE and

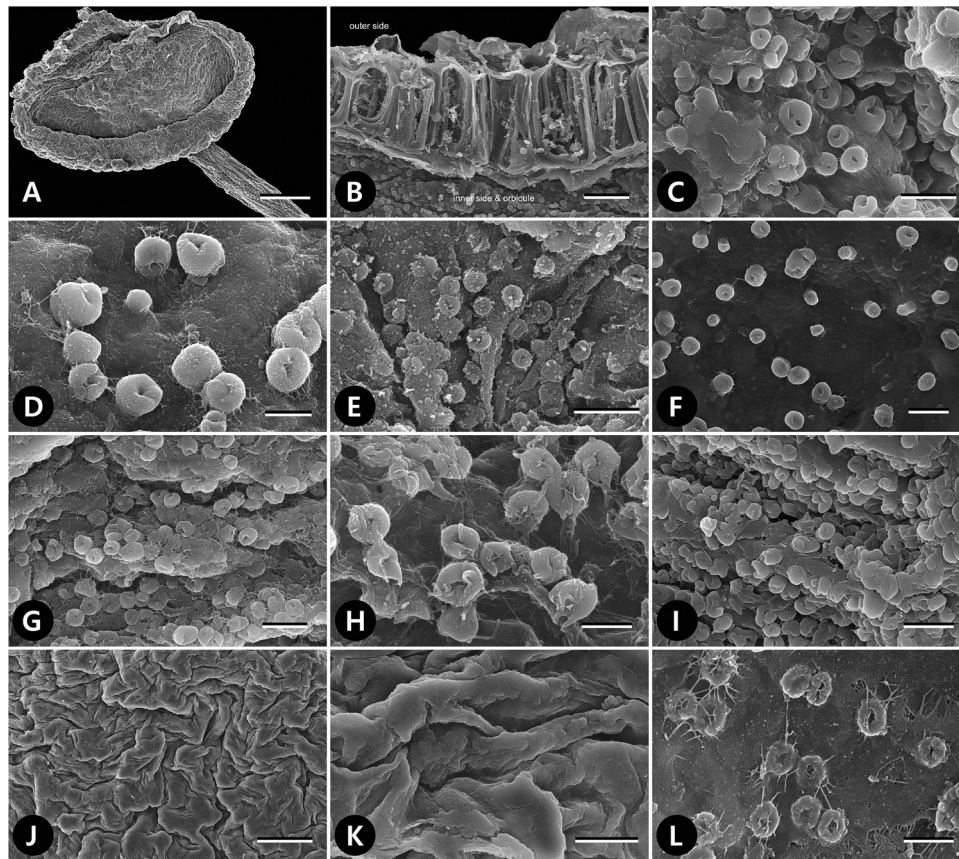


Figure 4. SEM micrographs of anther of Neillieae. (A), Anther with selected one thecae of *Neillia thibetica* var. *thibetica*. (B), Structure of the endothecium thickenings in cross section of *N. thibetica* var. *thibetica*. (C), *N. gracilis*: very abundant doughnut-shaped (ds) with psilate orbicules. (D, E). Abundant orbicules of ds with granulate. (D), *N. thibetica* var. *thibetica*. (E), *N. thyrsiflora*. (F), *N. uekii*: abundant orbicules of ds with psilate. (G–I), Very abundant ds with psilate and aggregated orbicules. (G), *Physocarpus amurensis*. (H), *P. malvaceus*. (I), *P. opulifolius*. (J–K), Inner locule wall with no traces of orbicules. (J), *P. insularis*. (K), *Spiraea chamaedryfolia*. (L), *Stephanandra incisa* var. *incisa*: abundant orbicules of ds with psilate. Scale bars: A, 100 µm; J, 20 µm; B, 10 µm; K, 5 µm; C, E, F, G, I, 2 µm; D, H, L, 1 µm.

the size range of ACE-examined pollen grains analysed in the present study agrees with previously published data (Naruhashi & Toyoshima, 1979; Chang, 1986; Hebda *et al.*, 1991; Lee *et al.*, 1993; Wang *et al.*, 1997; Zhou *et al.*, 1999). Moreover, colpus length was positively correlated with the length of the polar axis and with equatorial diameter, similar to that found in pollen grains of Sorbarieae (Song *et al.*, 2016).

Data on the shape of pollen grains are rather limited (Naruhashi & Toyoshima, 1979; Hebda *et al.*, 1991; Lee *et al.*, 1993; Zhou *et al.*, 1999), and *Neillia* pollen grains have been described as prolate–spheroidal in plants from Korea (P/E = 1.11; Lee *et al.*, 1993) and China (P/E = 1.07; Zhou *et al.*, 1999). In the present study, the majority of *Neillia* (65%) and *Stephanandra* (68%) pollen grains were oblate to oblate–spheroidal. However, prolate–spheroidal to prolate shapes were common in *Physocarpus* pollen

grains (75%; number of observed pollen/total number of pollen grains).

IMPLICATION OF SEXINE ORNAMENTATION CHARACTERS FOR SYSTEMATICS

The variation in sexine ornamentation proved to be a useful diagnostic characteristic in Neillieae. Three types of sexine ornamentation were defined: perforate; striate with macroperforations; and striate with microperforations. These patterns were consistently observed at the generic level. *Neillia* and *Stephanandra* had perforate ornamentation (Type I), whereas it was striate (Type II) in *Physocarpus*. Our results were consistent with the molecular phylogenetic tree of Oh & Potter (2005) and with the classification proposed by Oh (2016), who merged *Neillia* and *Stephanandra* into a single genus (Fig. 6). The differences found by Lee *et al.* (1993) in perforation

Table 4. The results of the principal component analysis (PCA) of the 12 quantitative pollen morphological characteristics of the tribe Neillieae and related species. The first four PCs with eigenvalue larger than one were represented. The components that were loaded most highly for each character are in bold

No.	PC	1	2	3	4
	Eigenvalue	6.283	3.030	0.991	0.760
	Variance cumulative (%)	52.36	77.61	85.87	92.20
	Characteristics*	Eigenvector			
1	P	-0.3369	-0.2861	-0.051	-0.1609
2	E	-0.3736	-0.1597	0.0860	0.0549
3	CL	-0.3187	-0.3204	0.0826	-0.1968
4	P/E	0.1146	-0.3055	-0.4157	-0.7288
5	CL/P	0.1142	-0.2232	0.8277	-0.3014
6	OB	-0.3539	-0.0823	0.1263	0.1400
7	WR	0.1716	-0.4709	-0.1256	0.2082
8	WV	0.1666	-0.4566	-0.2337	0.3141
9	PR	-0.3155	-0.0799	-0.0493	0.2886
10	EDPV	-0.3777	-0.1468	-0.0146	0.0474
11	AS	-0.3712	0.1509	-0.1130	0.0998
12	PAI	-0.2380	0.3987	-0.1532	-0.2309

* P, polar axis; E, equatorial diameter; CL, colpus length; P/E, polar axis/equatorial diameter; CL/P, colpus length/polar axis; OB, orbicules diameter; WR, width of ridge; WV, width of valley; PR, perforation diameter; EDPV, equatorial diameter in polar view; AS, apocolpus side; PAI, Polar Area Index.

diameter (*Neillia* spp.: c. 0.08 µm; *Stephanandra* spp.: c. 0.12 µm) and pollen shape between *Neillia* and *Stephanandra* species were not detected in the present study. In addition, pore size differences are known to occur within a taxon and perforation size variation has been found at the individual level (Remizowa *et al.*, 2008). The PCA biplot presented here also showed that species of *Neillia* and *Stephanandra* were clustered in the same group (Fig. 5). Therefore, the present palynological evidence supports the combination of *Stephanandra* and *Neillia* into one genus (Oh, 2006, 2016).

In their morphological study of *P. insularis*, Kim *et al.* (2000) suggested that this species belonged to *Spiraea chamaedryfolia* var. *ulmifolia*, as both species lacked stipules and follicular fruit dehiscence occurred only along the ventral suture. Potter *et al.* (2007) suggested that the lack of stipules and unitegmic ovules is potential synapomorphies in tribe Spiraeae. Oh *et al.* (2010) strongly suggested that *P. insularis* was not a species of *Physocarpus*, but a member of *Spiraea*, based on molecular phylogenetic results and in agreement with the morphological observations of Kim *et al.* (2000). However, Oh *et al.* (2010) did not agree that *P. insularis* should be placed in the synonymy with *Spiraea chamaedryfolia* var. *ulmifolia* (Kim *et al.*, 2000). Phylogenetic data showed that *P. insularis* is closely related to *S. chamaedryfolia* (including vars. *chamaedryfolia* and *ulmifolia*), but the morphology of *P. insularis* differs

from that of *S. chamaedryfolia*, as it has larger leaf blades, which are subcordate or truncate at the base, and a higher number of stamens (Oh *et al.*, 2010). Thus, *P. insularis* was transferred to *S. insularis* by Shin *et al.* (2011).

Although we could not find any palynological differences between *S. chamaedryfolia* and *P. insularis*, pollen features clearly supported the inclusion of *P. insularis* in *Spiraea*. In addition, the striate with microperforations sexine ornamentation is a general morphological characteristic of pollen of *Spiraea* spp. (Roh, 2010; Song *et al.*, 2017).

UTILIZATION OF ORBICULES IN SYSTEMATICS

Most orbicule studies have paid little attention to their intraspecific variability (Verstraete *et al.*, 2014). The constant presence of orbicules and the low intraspecific variability of their characters were confirmed in eight taxa, for which more than two specimens were examined. This observation suggested that orbicule characters might be stable at the species level.

The constant absence or presence of orbicules in most early diverging angiosperms [including the ANA groups (Amborellales, Nymphaeales and Austrobaileyales; APG IV, 2016)] is significant (Huysmans & Smets, 1998; Verstraete *et al.*, 2014). Because orbicules are consistently observed in tribe

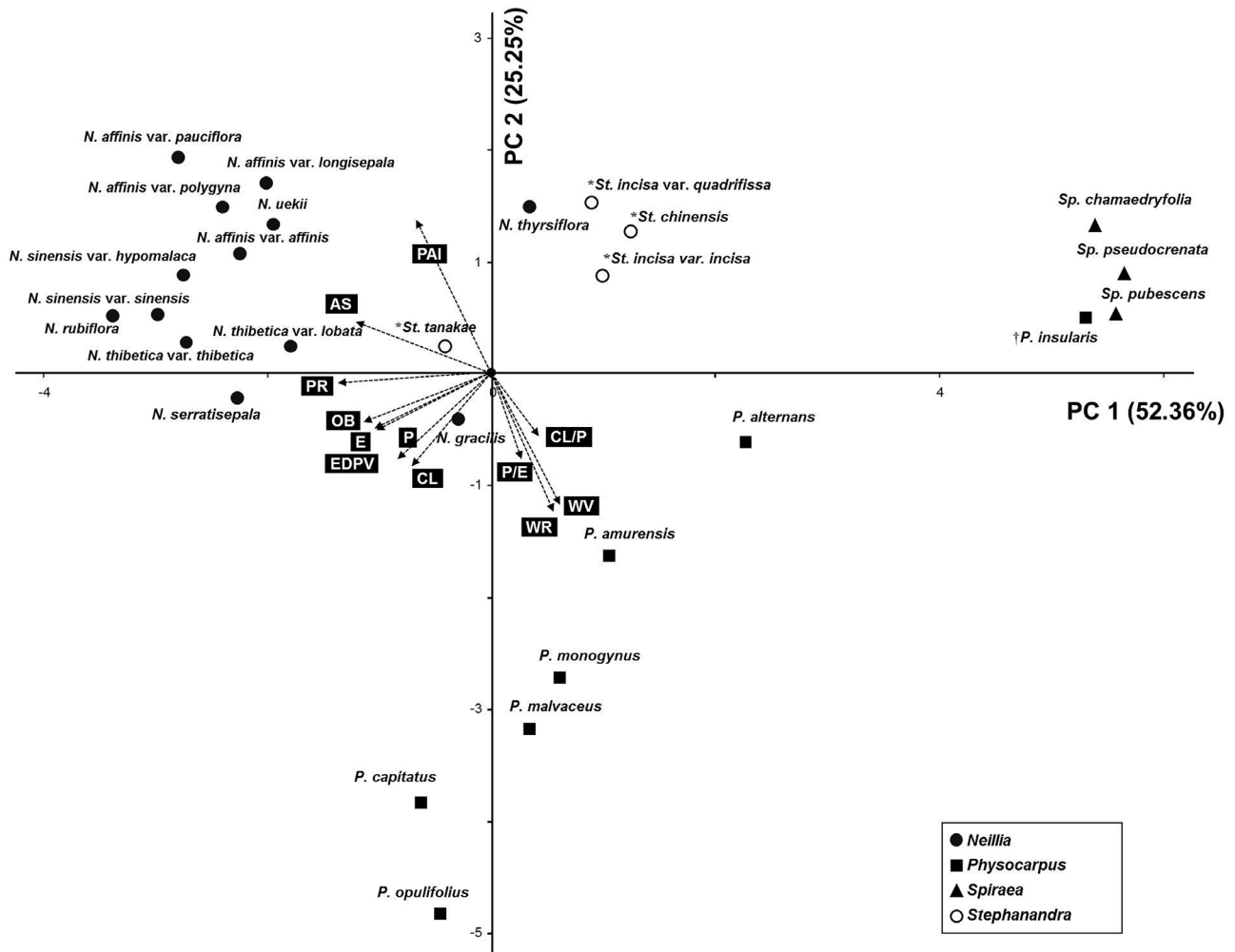


Figure 5. Principal component analysis (PCA) performed with the twelve pollen quantitative variables from tribe Neillieae and related species. AS, length of apocolpus side; CL, colpus length; CL/P, colpus length/polar axis; E, equatorial diameter; EDPV, equatorial diameter in polar view; OB, orbicule diameter; P, polar axis; P/E, polar axis/equatorial diameter; PAI, polar area index; PR, diameter of perforation; WR, width of ridge (muri); WV, width of valley (groove). Symbols †currently transferred from *Physocarpus* to *Spiraea* by Shin *et al.* (2011) and *proposed members of *Stephanandra* combined into *Neillia* by Oh (2006, 2016).

Sorbarieae (subfamily Amygdaloideae), the presence of orbicules could be an important characteristic in Rosaceae (Song *et al.*, 2016). Orbicules were also consistently found in Neillieae, exception in *P. insularis*. As the absence of orbicules might be a synapomorphic condition for *Spiraea* (tribe Spiraeae; Song *et al.*, 2017), orbicule absence also supports the transition of *P. insularis* to *Spiraea*.

Thus, the presence of orbicules might be of taxonomic value at the generic or tribal level in subfamily Amygdaloideae, but further studies of orbicule morphology in Rosaceae are needed to understand better their occurrence and evolutionary trends in angiosperms.

CONCLUSIONS

The sexine ornamentation and orbicule characteristics might have high systematic importance in Neillieae. Our results strongly supported the molecular phylogenetic hypothesis that *Stephanandra* and *Neillia* are a single genus and that *Physocarpus insularis* should be considered a member of *Spiraea* (Oh & Potter, 2005; Oh *et al.*, 2010). In addition, palynological traits might be useful for defining systematic groups at the generic or tribal level in Neillieae as they share the same palynological features. Pollen characteristics seem to be useful for reconstructing systematic relationships at the higher rank in Rosaceae. Further

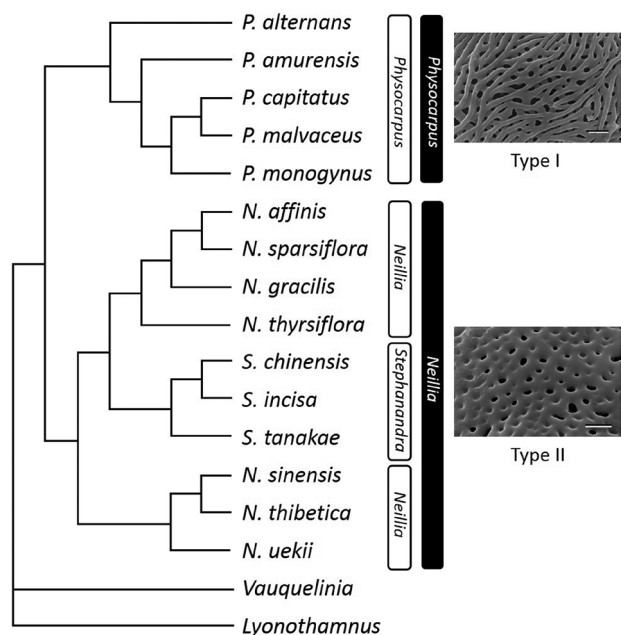


Figure 6. Mapping pollen sexine ornamentation types on the most recent molecular phylogenetic tree (adapted from Oh & Potter, 2005). The two major types of sexine ornamentation recognized were plotted on the topology. Open bars are indicated as traditional generic classification by Schulze-Menz (1964) and closed bars present a current generic system in tribe Neillieae by Oh (2006).

studies are necessary to evaluate the pollen variation in Amygdaloideae in a phylogenetic context.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix. Voucher specimens of the tribe Neillieae and related species that are examined in the present study.