

Numerical classification of *Rhododendron* (*Ericaceae*) based on seed morphology and protein profile

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Abstract. Seeds of 28 taxa and infra-specific taxa of *Rhododendron* were examined by light and electron microscopy and their protein patterns were determined by SDS-PAGE analysis. A combined data matrix was subjected to numerical analysis by Sørensen's similarity measure and Ward's clustering method to generate a dendrogram expressing the hierarchical phenetic relationships between the taxa. Two main groups have been recognized, of which one comprises two smaller groups, while the second is divided into three groups. Comparison between the five low-level groups and the subgenera in the two major traditional classifications of *Rhododendron* has shown that none of these subgenera is taxonomically secure. Infra-specific taxa of *Rh. brachycarpum* and *Rh. minus* adhered closely to each other, and the former species was isolated from the remaining taxa in one of the five low-level groups. Separation of *Rh. menziesii* into a genus (*Menziesia*) has not been supported and it seems best placed in the subgenus *Hymenanthes*.

Key Words: Classification, *Menziesia*, *Rhododendron*, seed morphology, seed protein profile

Introduction

Rhododendron L. (*Ericaceae*) is one of the largest genera of flowering plants. It includes about 1215 species and subspecies divided into nine subgenera (Sleumer 1949, Chamberlain & al. 1996, Gibbs & al. 2011, Argent 2015). The genus is distributed across the Northern Hemisphere and Southeast Asia to Oceania, and centered in Asia (Fang & al. 2005; Wang & al. 2014), between 65° N to 20° S in tropical, temperate and subtemperate zones. It occurs at altitudes from few hundred to about 5500 m a.s.l.

According to Chamberlain & al. (1996) and Argent (2015), *Rhododendron* is classified into nine subgenera: *Azaleastrum*, *Candidastrum*, *Hymenanthes*, *Mumeazalea*, *Pentanthera*, *Rhododendron*, *Therorhodium*, *Tsutsusi*, and *Vireya*. RAPD analysis, chloroplast DNA markers, DNA barcoding and other molecular research into the taxonomic structure of *Rhododendron* largely support the phenetic taxonomy, except for some sections of *Vireya* (Zhou & al. 2009, De Key-

ser & al. 2010; Milne & al. 2010, Kutsev & Karakulov 2011). Other molecular works have reached different conclusions on the arrangement of sections into subgenera (Kurashige & al. 1998, 2001; Goetsch & al. 2005; Zhou & al. 2009; Kron & Powell 2009; Milne & al. 2010; Craven & al. 2011; Tsai & al. 2012; Yan & al. 2015). In spite of the number of studies on *Rhododendron* taxonomy, the range of taxa used in these studies was not sufficient to confirm or revise the morphologically based infrageneric classification of the genus. Phylogeny established by Goetsch & al. (2005) was based on species from all higher-level infrageneric groups of *Rhododendron* and supported that of Sleumer (1949) over that of Chamberlain & al. (1996).

In view of the wide-scale discrepancies between the conflicting arrangements of the species into subgenera, sections and subsections, the present study was undertaken to reassess the taxonomic worth of these arrangements by numerical analysis of the comparative data for seed morphology and protein profiles of the species.

Material and methods

Seeds of 28 *Rhododendron* taxa (25 species with five subspecies and two varieties) were obtained from the botanical gardens and herbaria of the University of British Columbia (UBC), Canada, the Dawes Arboretum (DAWES), United States of America, and the Polish Academy of Sciences (Table 1). Seeds were examined by light microscope (LM) and six mature seeds of each taxon were selected for micro-structure studies using scanning electron microscope (SEM). Seeds were mounted on SEM stubs by double-sided tape, coated with gold palladium in a vacuum evapo-

Table 1. Scientific names with their abbreviations (Abbr.) and sources of seeds of 25 species with five subspecies and two varieties of *Rhododendron*; names and abbreviations are in alphabetical order.

Abb.	Taxa	Source of seeds
Albiflor	<i>Rh. albiflorum</i> Hook.	Canada
Arboresc	<i>Rh. arborescens</i> (Pursh) Torr.	USA
Brach.br	<i>Rh. brachycarpum</i> D.Don ex G.Don subsp. <i>brachycarpum</i>	Poland
Brach.fa	<i>Rh. brachycarpum</i> subsp. <i>faurieri</i> (Franch.) D.F.Chamb.	Poland
Brach.ro	<i>Rh. brachycarpum</i> var. <i>roseum</i> Koidz.	Poland
Calendul	<i>Rh. calendulaceum</i> (Michx.) Torr.	Poland & USA
Canadens	<i>Rh. canadense</i> (L.) Torr.	Poland
Catawbie	<i>Rh. catawbiense</i> Michx.	Poland & USA
Degronia	<i>Rh. degronianum</i> subsp. <i>yakushmanum</i> (Nakai) H.Hara	Poland
Dichroan	<i>Rh. dichroanthum</i> subsp. <i>scyphocalyx</i> (Balf.f. & Forrest) Cowan	Poland
Kaempfer	<i>Rh. kaempferi</i> Planch.	Poland
kKiusianu	<i>Rh. kiusianum</i> Makino	Poland
Luteum	<i>Rh. luteum</i> Sweet	Poland
Macrophy	<i>Rh. macrophyllum</i> D.Don ex G.Don	Poland
Makinoi	<i>Rh. makinoi</i> Tagg.	Poland
Maximum	<i>Rh. maximum</i> L.	Poland
Menziesi	<i>Rh. menziesii</i> Craven	Canada
Minus	<i>Rh. minus</i> Michx.	USA
Minus.mi	<i>Rh. minus</i> Michx. subsp. <i>minus</i>	Poland
Periclym	<i>Rh. periclymenoides</i> (Michx.) Shinnars	Poland
Ponticum	<i>Rh. ponticum</i> L.	Poland
Prinophy	<i>Rh. prinophyllum</i> (Small) Millais	Poland & USA
Reticula	<i>Rh. reticulatum</i> D.Don ex G.Don	Poland
Smirnowi	<i>Rh. smirnowii</i> Trautv. ex Regel	Poland
Vaseyi	<i>Rh. vaseyi</i> A.Gray	Poland
Viscosum	<i>Rh. viscosum</i> (L.) Torr.	Poland
Wardii	<i>Rh. wardii</i> W.W.Sm.	Poland
Yedoense	<i>Rh. yedoense</i> var. <i>poukhanense</i> Maxim. ex Regel	Poland

rator, examined and photographed with a JEOL JSM 5400 LV scanning electron microscope, which operated with accelerated voltage of 15 KV at an electron microscopy unit, Assiut University, Egypt. Since testa cell morphology varies depending on the region of the examined seeds, close-up views were always taken from the lateral region of the seed (Barthlott & Voit 1979). Terminology concerning the description of outer seed patterns follows Barthlott (1981 & 1990) and Stearn (1992). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for total proteins of the seeds of 28 *Rhododendron* taxa, according to the method of Laemmli (1970), as modified by Studier (1973). All gels were scanned and analyzed using the Gel Doc Vilber Lourmat system.

Variations in seed macro- and micro-morphology were defined as eight distinct characters, with a total of 22 character-states. The appropriate states of the eight characters were recorded for each of the 28 *Rhododendron* taxa in a data matrix, along with the presence/absence of protein bands in the profile of every taxon. The data matrix was subjected to numerical analysis using six radically different combinations of dissimilarity measures and clustering methods available in the program package PC-Word version 5 (McCune 1997) for Windows. Only the combination of Sørensen's measures and Ward's clustering method was selected for further discussion, because it yielded a dendrogram, which had the lowest chaining percentage 4.46% (i.e. the optimum clustering intensity), and the assemblages of taxa had the closest resemblance to traditional classifications of the genus into subgenera. Program specifications called for the taxa names abbreviations as shown in Table 1.

Results

The following list of characters sums up the different aspects of variation in the seed morphological characters observed in 28 *Rhododendron* taxa included in the present study. The states of characters 1–5 are illustrated in Fig. 1, and those of characters 6–7 in Fig. 2:

1. Seed shapes: rod-shaped 1/ oblong 2/ fusiform 3/ ovate 4/ rectangular 5/ irregular 6
2. Seed end: pointed 1/ rounded 2/ flattened 3

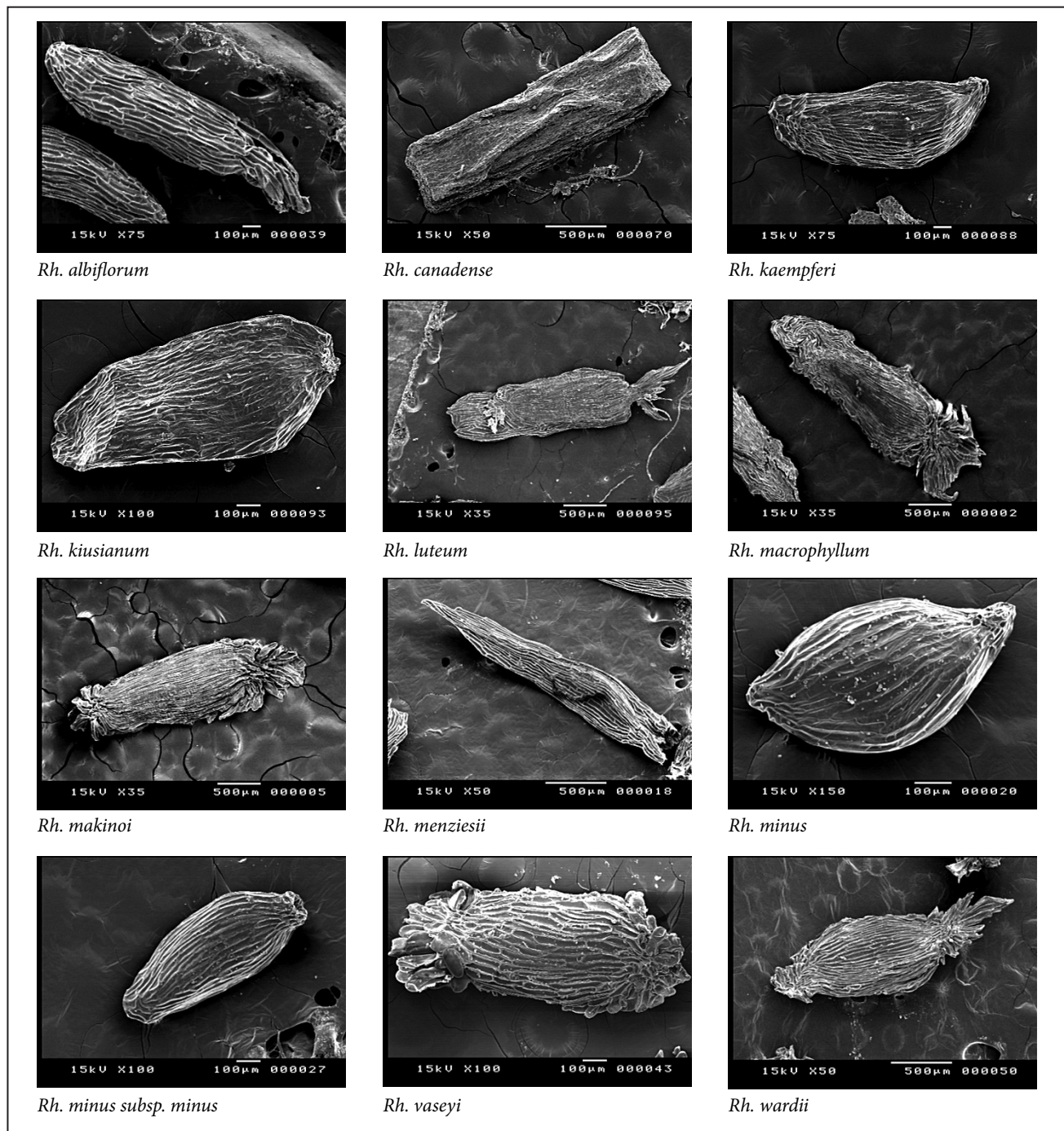


Fig. 1. SEM micrographs of mature seeds of 12 *Rhododendron* taxa, showing their gross morphology.

3. Crown at seed ends: at one end 1/ at both ends 2/ absent 3
4. Seed length: 1.0 -1.4 mm 1/ 1.41- 2.5 mm 0
5. Seed width: 0.2 – 0.5 mm 1/ 0.51 – 1.0 mm 0
6. Seed surface: reticulate 1/ irregular 0
7. Cell shape: extended polygonal 1/ irregular 2/ rectangular 3
8. Seeds: brown 1/ yellow 0.

A numerical analysis of the combined data matrix has resulted in the phenogram shown in Fig. 3. Distribution of the six different seed shapes among the 28 taxa was as follows:

1. Rod-shaped in *Rh. albiflorum*, *Rh. brachycarpum* subsp. *brachycarpum*, *Rh. menziesii*, *Rh. prinophyllum*, *Rh. smirnowii*, *Rh. degronianum* subsp. *yakushmanum*

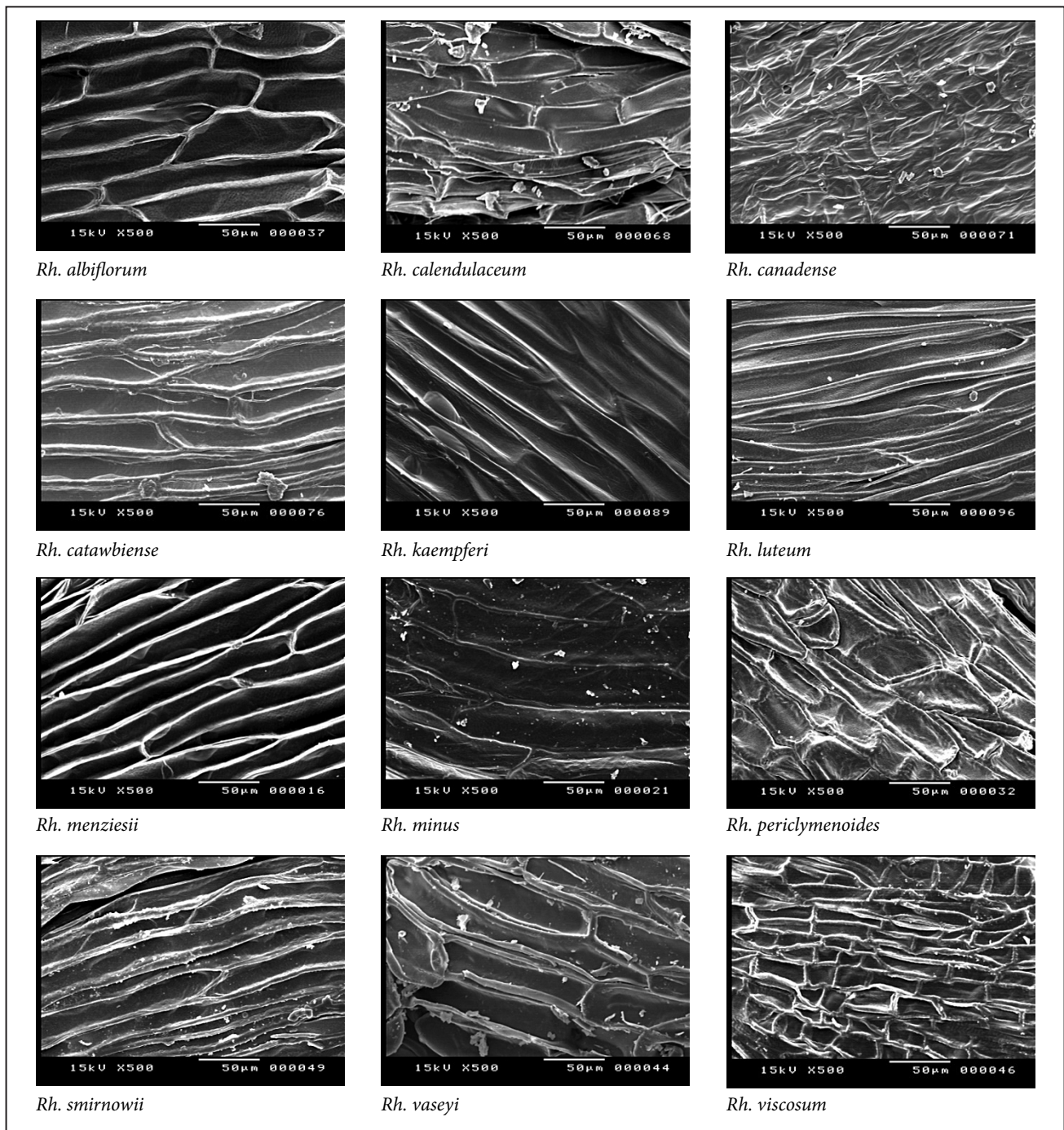


Fig. 2. SEM micrographs of mature seeds of 12 *Rhododendron* taxa showing their patterns of surface ornamentation.

2. Oblong in *Rh. arborescens*, *Rh. calendulaceum*, *Rh. luteum*, *Rh. minus* subsp. *minus*, *Rh. ponticum*, *Rh. vaseyi*.
 3. Fusiform in *Rh. brachycarpum* subsp. *faurieri*, *Rh. catawbiense*, *Rh. kaempferi*, *Rh. kiusianum*, *Rh. maximum*, *Rh. reticulatum*.
 4. Ovate in *Rh. brachycarpum* var. *roseum*, *Rh. dichroanthum* subsp. *scyphocalyx*, *Rh. macrophyllum*, *Rh. makinoi*, *Rh. minus*, *Rh. wardii*, *Rh. yedoense* var. *poukhanense*.
 5. Rectangular only in *Rh. canadense*.
 6. Irregular in *Rh. periclymenoides* and *Rh. viscosum*.
- The seed end was flat in only one species (*Rh. canadense*), pointed in 17 taxa and rounded in the remaining 10 taxa. Crown was recorded at both seed

ends in five species (*Rh. makinoi*, *Rh. maximum*, *Rh. ponticum*, *Rh. vaseyi* and *Rh. degroonianum* subsp. *yakushmanum*), at one end in 11 species, and was completely absent in 12 species. Only five species (*Rh. albiflorum*, *Rh. canadense*, *Rh. luteum*, *Rh. menziesii* and *Rh. prinophyllum*) had yellow seeds, while the remaining taxa had brown seeds. Seed length varied from 0.1–1.4 mm in 11 species and from 1.41–2.5 mm in 17 species. Seed width ranged between 0.2–0.5 mm in ten species and from 0.51 mm to 1 mm in the remaining 18 species. Overall seed ornamentation appeared reticulate in all species, except for *Rh. arborescens*, where it was irregular. Cell shape was extended polygonal in 20 taxa, rectangular in six taxa (*Rh. calendulaceum*, *Rh. menziesii*, *Rh. minus*, *Rh. minus* subsp. *minus*, *Rh. periclymenoides* and *Rh. viscosum*), and irregular in only two species (*Rh. arborescens*, *Rh. reticulatum*).

Seed protein profiling showed a total of 11 bands with various distribution among the 28 taxa. Molecular masses of these bands ranged from 147 to 8.666 kDa. Only eight bands were unique in seven taxa: *Rh. luteum* had two specific bands (147 kDa and 122.2 kDa), while *Rh. smirnowii*, *Rh. kaempferi*, *Rh. minus*, *Rh. catawbiense*, *Rh. prinophyllum* and *Rh. menziesii* had each a single specific band (111.7 kDa, 43.09 kDa, 36.51 kDa, 36.51 kDa, 33.44 kDa, and 10.28 kDa, re-

spectively). These bands could be regarded as specific markers for distinguishing each taxon from the rest. All other bands were polymorphic. Distribution of seed morphological characters and protein bands among the 28 *Rhododendron* taxa could be used in reconstruction of the original data matrix.

The dendrogram in Fig. 3 indicates clearly that the 28 *Rhododendron* taxa are divided into two main groups A and B. Group A is further divided into two subordinate groups (AC and AD), while Group B comprises three smaller assemblages (BE, BF and BG). The species composition of the five low-level groups is as follows:

AC (5 taxa): *Rh. albiflorum* Hook., *Rh. kaempferi* Planch., *Rh. arborescens* (Pursh) Torr., *Rh. calendulaceum* (Michx.) Torr., *Rh. canadense* (L.) Torr.

AD (3 taxa): *Rh. brachycarpum* D.Don ex G.Don subsp. *brachycarpum*, *Rh. brachycarpum* subsp. *faurieri* (Franch.) D.F.Chamb., *Rh. brachycarpum* var. *roseum* Koidz.

BE (6 taxa): *Rh. catawbiense* Michx., *Rh. dichroanthum* subsp. *scyphocalyx* (Balf.f. & Forrest) Cowan, *Rh. maximum* L., *Rh. ponticum* L., *Rh. degroonianum* subsp. *yakushmanum* (Nakai) H.Hara, *Rh. menziesii* Craven

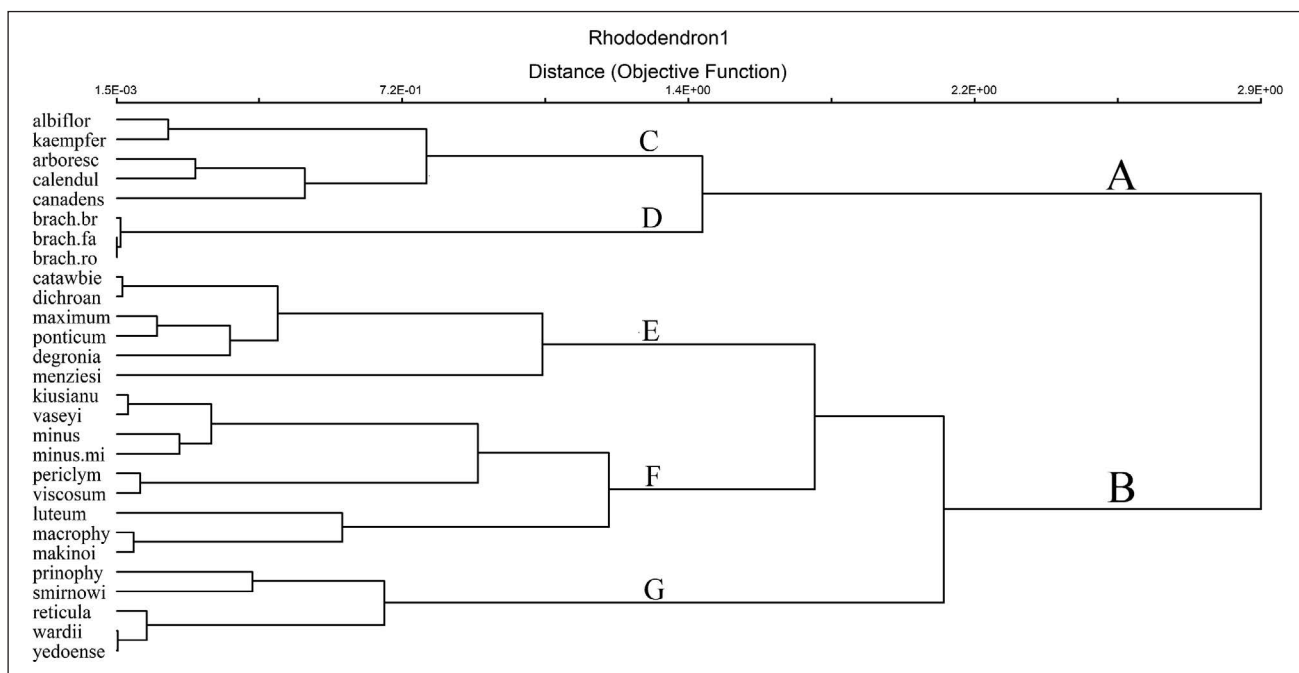


Fig. 3. Dendrogram illustrating the hierarchical phenetic relationships between 28 species and infra-specific taxa of *Rhododendron* based on numerical analysis of characters of seed morphology and protein patterns, using a combination of the Sørensen's measures of similarity and Ward's clustering method; the chaining percentage = 4.46.

BF (9 taxa): *Rh. kiusianum* Makino, *Rh. vaseyi* A.Gray, *Rh. minus* Michx., *Rh. minus* Michx. subsp. *minus*, *Rh. periclymenoides* (Michx.) Shinnery, *Rh. viscosum* (L.) Torr., *Rh. luteum* Sweet, *Rh. macrophyllum* D.Don ex G.Don, *Rh. makinoi* Tagg.

BG (5 taxa): *Rh. prinophyllum* (Small) Millais, *Rh. smirnowii* Trautv. ex Regel, *Rh. reticulatum* D.Don ex G.Don, *Rh. wardii* W.W.Sm., *Rh. yedoense* var. *poukhanense* Maxim. ex Regel

Discussion

The hierarchical taxonomic arrangement of the 28 *Rhododendron* taxa in Fig. 3 is set against the subgeneric disposition of these taxa in the two traditional classifications of the genus by Chamberlain & al. (1996) and Goetsch & al. (2005) in Table 2. The most striking result is that none of the six subgenera recognized in the two classifications (*Azaleastrum*, *Candidastrum*, *Hymenanthes*, *Pentanthera*, *Rhododendron*, and *Tsutsusi*) has emerged intact in the present study.

In the classification by Chamberlain & al. (1996), the two species representing subgenus *Candidastrum* (*Rh. albiflorum* and *Rh. menziesii*) were widely separated in the groups AC and BE. Similarly, the three species representing subgenus *Tsutsusi* (*Rh. kaempferi*, *Rh. kiusianum* and *Rh. reticulatum*) were distributed in three different groups (AC, BE and BG respectively). Representatives of the large subgenus *Pentanthera* were found in groups AC, BF and BG, whereas those of subgenus *Hymenanthes* were scattered across all five low-level groups in Fig. 3, except for group AC. The two largest subgenera in the classification by Goetsch & al. (2005) suffered similar disruption in Table 2: *Azaleastrum* in three groups (AC, BE and BF and BE) and *Hymenanthes* in all five low-level groups. Clearly, the sub-generic concept in the two major traditional classifications of *Rhododendron* was far from being taxonomically solid. In contrast, infra-specific taxa of *Rh. brachycarpum* and *Rh. minus* adhered to each other in groups AD and BF, respectively (Fig. 3 and Table 2).

While the three infra-specific taxa of *Rh. brachycarpum* are closely related to each other at least in terms of the recorded characters of seed morphology and seed protein profile, they seem deeply distinct from the rest of *Rhododendron* species, be-

cause they are consistently isolated in a group of their own (group AD) in all six numerical analyses performed in the present study (only one dendrogram is shown in Fig. 3).

Recognition of *Rh. menziesii* along with nine other *Rhododendron* species as a separate genus, *Menziesia* Smith, was not supported by the present results, which indicated its close affinity to other species of subgenus *Hymenanthes* in group BE. Further support for submerging *Menziesia* into *Rhododendron* came from some morphological and an-

Table 2. Comparison between the hierarchical arrangement of 28 *Rhododendron* taxa in Fig. 3 and their subgeneric disposition in the two classification systems of Chamberlain & al. (1996) and Goetsch & al. (2005).

Taxa	Fig. 3	Chamberlain & al. (1996)	Goetsch & al. (2005)
<i>Rh. albiflorum</i> Hook.		Candidastrum	Azaleastrum
<i>Rh. kaempferi</i> Planch.		Tsutsusi	Azaleastrum
<i>Rh. arborescens</i> (Pursh) Torr.	AC	Pentanthera	Hymenanthes
<i>Rh. calendulaceum</i> (Michx.) Torr.		Pentanthera	Hymenanthes
<i>Rh. canadense</i> (L.) Torr.		Pentanthera	Hymenanthes
<i>Rh. brachycarpum</i> D.Don ex G.Don subsp. <i>brachycarpum</i>		Hymenanthes	Hymenanthes
<i>Rh. brachycarpum</i> subsp. <i>faurieri</i> (Franch.) D.F.Chamb.	AD	Hymenanthes	Hymenanthes
<i>Rh. brachycarpum</i> var. <i>roseum</i> Koidz.		Hymenanthes	Hymenanthes
<i>Rh. catawbiense</i> Michx.		Hymenanthes	Hymenanthes
<i>Rh. dichroanthum</i> subsp. <i>scyphocalyx</i> (Balf.f. & Forrest) Cowan		Hymenanthes	Hymenanthes
<i>Rh. maximum</i> L.		Hymenanthes	Hymenanthes
<i>Rh. ponticum</i> L.	BE	Hymenanthes	Hymenanthes
<i>Rh. degronianum</i> subsp. <i>yakushimanum</i> (Nakai) H.Hara		Hymenanthes	Hymenanthes
<i>Rh. menziesii</i> Craven		Candidastrum	Azaleastrum
<i>Rh. kiusianum</i> Makino		Tsutsusi	Azaleastrum
<i>Rh. vaseyi</i> A.Gray		Pentanthera	Azaleastrum
<i>Rh. minus</i> Michx.		Rhododendron	Rhododendron
<i>Rh. minus</i> Michx. subsp. <i>minus</i>		Rhododendron	Rhododendron
<i>Rh. periclymenoides</i> (Michx.) Shinnery	BF	Pentanthera	Hymenanthes
<i>Rh. viscosum</i> (L.) Torr.		Pentanthera	Hymenanthes
<i>Rh. luteum</i> Sweet		Pentanthera	Hymenanthes
<i>Rh. macrophyllum</i> D.Don ex G.Don		Hymenanthes	Hymenanthes
<i>Rh. makinoi</i> Tagg.		Hymenanthes	Hymenanthes
<i>Rh. prinophyllum</i> (Small) Millais		Pentanthera	Hymenanthes
<i>Rh. smirnowii</i> Trautv. ex Regel		Hymenanthes	Hymenanthes
<i>Rh. reticulatum</i> D.Don ex G.Don	BG	Tsutsusi	Azaleastrum
<i>Rh. wardii</i> W.W.Sm.		Hymenanthes	Hymenanthes
<i>Rh. yedoense</i> var. <i>poukhanense</i> Maxim. ex Regel		Tsutsusi	Azaleastrum

atomical studies (e.g. Copeland 1943; Fang & al. 2005; Craven 2011), numerous phylogenetic works based on DNA sequencing data (e.g. Kurashige & al. 2001; Stevens & al. 2004; Goetsch & al. 2005; Craven 2011), and successful experiments of intergeneric hybridization carried out by Handa & al. (2003 & 2006) and Kita & al. (2005). Therefore, in the light of the present results, it has seemed plausible to assume that if any of the species in the present sample were a candidate for the transfer from *Rhododendron* to another genus, it was *Rh. brachycarpum* and not *Rh. menziesii*.

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