10.21608/TAEC.2022.138652.1037

Taeckholmia 42 (2022): 1-11

Taxonomic significance of seed characters and SDS-PAGE analysis in the classification of Ericaceae

Lamiaa F. Shalabi*, Nagwan Hamdy and Ethar A. Hussein

Department of Biological and Geological Sciences, Faculty of Education, Ain Shams University, Cairo, Egypt.

* Corresponding author: Flora_lamiaa@yahoo.com

Abstract

Classification of nineteen taxa, belong to ten genera of family Ericaceae are studied. The study based on macro-, micro-morphological characters of seeds and SDS-PAGE analysis techniques. The phenetic relationships of the studied taxa were expressed by UPGMA-clustering method using NTSYS-pc 2.2 software. The UPGMA phenogram based on 47 characters revealed the separation of two major clusters (A) and (B). Group (A) subdivided into two sub ordinary clusters (AC), expressed subfamily Vaccinoideae, and (AD) which expressed together with main group (B) subfamily Ericoideae. The studied genera are distributed equally between these two subfamilies. Vaccinoideae is represented by five tribes: Vaccinieae, Gaultherieae, Oxydendreae, Lyonieae and Andromedeae. Ericoideae is separated as two clades representing two tribes: (AD) Phyllodoceae and (B) Rhodoreae. The produced hierarchical taxonomic arrangement typically matches the traditional classifications of the family. Clustering of *Menziesia pilosa* with *Rhododendron menziesii* in near distance with all *Rhododendron* taxa confirmed the placement of both genera under tribe Rhodoreae, and supports the transfer of genus *Menziesia* to be nested in *Rhododendron* as recommended by some recent cladistics analyses of DNA data.

***Keywords*:** Classification, Ericaceae, SDS-PAGE analysis, Seed morphology, Phenogram

Introduction

Ericaceae is a large family widely distributed in temperate and subarctic regions, also at high tropical altitudes. The family includes many herbs, dwarf shrubs, shrubs and trees, some of them are economically important i.e, Cranberry, Blueberry, Huckleberry and *Rhododendron* (Christenhusz & Byng, 2016).

Ericaceae are hardy plants, they are able to live in the environmental extremes of mountain tops, arctic conditions, tree trunks, branches, swamps, volcanoes, rocks and acid oligotrophic soil which is often sandy or peaty. However, apart from a few saprophytic members need high light levels and many need a good supply of moisture (Vander 1983; Heads 2003).

The taxonomy of the Ericaceae is notoriously controversial from family level down, However, DNA results are now appearing for many groups, and these give variation in taxonomy (Olson 1980; Heads 2003).

Ericaceae includes 125 genera and 4000 species (Fang et al. 2005 and Fagundez & Izco 2004), while Christenhusz & Byng (2016) classified into124 genera and 4250 species. Chamberlain et al. (1996) and Fang et al. (2005) stated that *Rhododendron* is a very large genus in Ericaceae, includes near 1000 species, followed by genus *Erica* which includes 860 species in the world. *Rhododendron* and *Erica* are ecologically important and widely cultivated in Europe, but they are not highly specious there (Fraga 1984; Takahashi 1993; Heads 2003), most taxa of these two genera are cultivated in temperate and sub-temperate regions as ornamentals (Craven et al. 2008).

Seeds of Ericaceae were the subject of many studies, mostly germination (Pons 1989) or regeneration of heathlands (Granstrom 1987; Barclay-Estrup & Gimingham 1994). Recently, seed morphology is used in solving some systematic problems in some genera as: *Erica* (Fagundez & Izco 2003a, 2003b, 2011), *Calluna* (Fagundez & Izco 2004), *Rubusgeoides* (Fredes et al. 2016) and *Rhododendron* (Shalabi et al. 2020).

Present study aims to revise the taxonomic relationships within family Ericaceae using macro- and micro-morphological characters of seeds as recorded by LM and SEM, as well as, SDS-PAGE analysis techniques.

Materials and methods

Plant sample

The study included 19 taxa belong to 10 genera. Seeds of the selected taxa were collected between (2016- 2018) from United States of America,

Received on 16/05/2022 and accepted on 11/07/2022

Edited by Prof. Dr. Rim Hamdy

Canada and Poland, through different botanical gardens, herbaria and arboreta A list of taxa and collection data is given in Table (1).

Seed Scanning Technique

Seeds examined by Light Microscope (LM) for the study of external morphology of seeds (seed colors are observed by naked eye). For Scanning Electron Microscopy (SEM) studies, four mature seeds were selected from each taxon. The seeds were mounted on SEM stubs, using double sided cellotape, coated with gold, palladium in vacuum evaporator, examined and photographed in a JEOL JSM 5400 LV scanning electron microscope which operated at accelerated voltage of 15 KV, at electron microscopy unit, Assiut University, Egypt. Since testa cell morphology varies depending on the region examined, close-up views were always taken from the lateral region of the seed (Barthlott & Voit 1979). Terminology used in the description of the outer seed pattern follows Barthlott & Hunt (2000).

Table (1): Scientific names of 19 taxa, belong to 10 genera of Ericaceae and sources of seeds from the botanical gardens and herbaria of the University of British Columbia (UBC), Canada; The Dawes Arboretum (DAWES), United States of America; and Polish Academy of Sciences (PAN). * Data concerning these taxa (seed scan and protein) had been published by Shalabi et al. (2020)

No.	Taxa	Source of seeds
1	*Chamaedaphne calyculata* (L.) Moench	(DAWES)
2	*Elliottia pyroliflora* (Bong.) Brim & P.F.Stevens	(UBC)
3	*Kalmia latifolia* L.	(DAWES)
4	*Menziesia pilosa* (Michx.) Juss.	(DAWES)
5	*Oxydendrum arboretum* (L.) DC.	(DAWES)
6	*Phyllodoce empetriformis* (Sm.) D.Don	(UBC)
7	*Pieris floribunda* (Pursh) Benth. & Hook. f.	(DAWES)
8	*Rhododendron kiusianum* Makino*	(PAN)
9	*Rhododendron luteum* Sweet*	(PAN)
10	*Rhododendron macrophyllum* D.Don ex G.Don*	(PAN)
11	*Rhododendron makinoi* Tagg. ex Nakai *	(PAN)
12	*Rhododendron maximum* L. *	(PAN)
13	*Rhododendron menziesii* Craven*	(UBC)
14	*Rhododendron minus* var. *chapmanii* (Alph.Wood) W.H.Duncan & Pullen*	(DAWES)
15	*Rhododendron minus* Michx. var. *minus* *	(PAN)
16	*Rhododendron periclymenoides* (Michx.) Shinners*	(PAN)
17	*Rhododendron ponticum* L. *	(PAN)
18	*Vaccinium ovalifolium* Sm. in Rees	(UBC)
19	*Zenobia pulverulenta* (W. Bartram ex Willd.) Pollard	(DAWES)

SDS-Protein analysis technique

Seed protein were analyzed using discontinuous polyacrylamide gel electrophoresis method in the presence of sodium dodecylesulphate (i.e., DISC-SDS-PAGE) in 10 % acrylamide slab gels following the method of Laemmli (1970). Extraction of proteins was performed by mixing 0.02 g of seeds with an equal weight of pure, clean, sterile fine sand. The seed were ground to fine powder using a mortar and pestle and homogenized with 1 M Tris-HCl buffer (pH 8.8) in clean Eppendorf tube and left in refrigerator overnight. The liquor was centrifuged at 10.000 rpm for 10 min. and the supernatant was kept in deep-freeze unit use for electrophoresis. 30 ml of protein extract was added to an equal volume of the treatment buffer (pH 6.8) and boiled for 10 minutes in water bath before loading in the gel. The gel buffer that was replaced by 0.5 M Tris-HCl buffer (pH 6.8). This was prepared by dissolving 6.05 g Tris in 50 ml distilled water by using a magnetic stirrer and the pH was adjusted to 6.8 by conc. HCl. The volume was completed to 100 ml with distilled water and kept at 4EC. The Staking gel (4%) was prepared by mixing 1.25 ml 1M Tris-HCl (pH 6.8); 1.7 ml acrylamide; 6.8 ml distilled water. Degassed and then 0.1 ml 10 % freshly prepared Ammonium persulphate, 0.1 ml 10 % SDS and 10 ml TEMED were added. Staining solution was prepared by dissolving 1g Commassie blue R-250, 455 ml Methanol, 90 ml Glacial acetic acid and 455 ml Distilled water.

Shacked well with a magnetic stirrer and preserved at 4°C until use. Gels were stained with such solution overnight with gently agitation. The gel was distained after the appearance of bands and photographed. All gels were scanned and analyzed.

Data analysis

For preparing the raw data matrix, a combination of all characters (seed morphology and protein pattern) which recorded as multistate characters, were changed into binary characters and coded as 1 and 0 for presence and absence respectively (Table 2 & 3). NTSYS- pc 2.2 software program (Rohlf

2005) was used in the data analysis by: the raw data matrix was standardized with STAND module; similarity matrix was generated by SIMQUAL module based on Jaccard coefficient. Clustering was established using Un-weighted Pair-Group Method with Arithmetic average (UPGMA) and represented in phenogram (tree). The distortion between each tree and its related distance matrix (Rohlf & Sokal 1981) was evaluated by computing the tree's cophenetic (ultramatric) value matrix using COPH and comparing them using MXCOMP modules.

Results

According to Table (1), the study includes 19 taxa of Ericaceae, ten out of them are species and varieties of *Rhododendron.* Recording the macro- and micro-morphological features of seeds composed up total 11characters with 27-character states (character list, Table 2). The states of characters 1–6 are illustrated in Figure (1) representing the macro-morphological seed characters, and those of characters 7–11 in Figure (2), representing the micro-morphological characters.

Six taxa are characterized by the ovate seed shape, seven are with oblong seed shape, and four taxa are with spindle seed shape, while seeds of only one species *Rhododendron menziesii* are recorded with rod, needle shape and other one *Rhododendron periclymenoides* with irregular seed shape. Seed end status divided the studied taxa into two groups: 8 taxa with pointed seed end, while the remaining 11 taxa are with rounded seed end. Concerning the seed measurements, 12 taxa reported seed lengths between 0.1 - 1.4 mm, while the remaining 7 taxa with longer seeds (1.41 - 2.6 mm).

Table (2): Character list used in the delimitation of the Ericaceae taxa based on seed morphology. For combined data matrix, see Appendix

	Characters	No.	Character states
1	Seed shape	**1**	Rod, needle
		2	Oblong
		3	Spindle
		4	Ovate
		5	Irregular
2	Seed end	**6**	Pointed
		7	Rounded
3	Seed Length (L) mm	**8**	0.1 - 1.4
		9	1.41 -2.6
4	Seed Width (W) mm	**10**	0.2 - 0.5
		11	0.51 -1
5	L/W Ratio	**12**	0.3 - 2.3
		13	2.31 - 3.6
		14	3.61 - 8.9
6	Color	**15**	Brown
		16	Yellow
7	Cell Length (L) mm	**17**	≤ 0.12
		18	≥ 0.121
8	Cell Width (W) mm	**19**	≤ 0.013
		20	> 0.013
9	Cell elongation Coefficient (P)= L/W	**21**	0.5 -12
		22	12.1 -22
10	Cell Shape	**23**	Extended polygonal
		24	Hexagonal
		25	Rectangular
11	Anticlinal Wall Thickness mm	**26**	≤ 0.005
		27	> 0.005

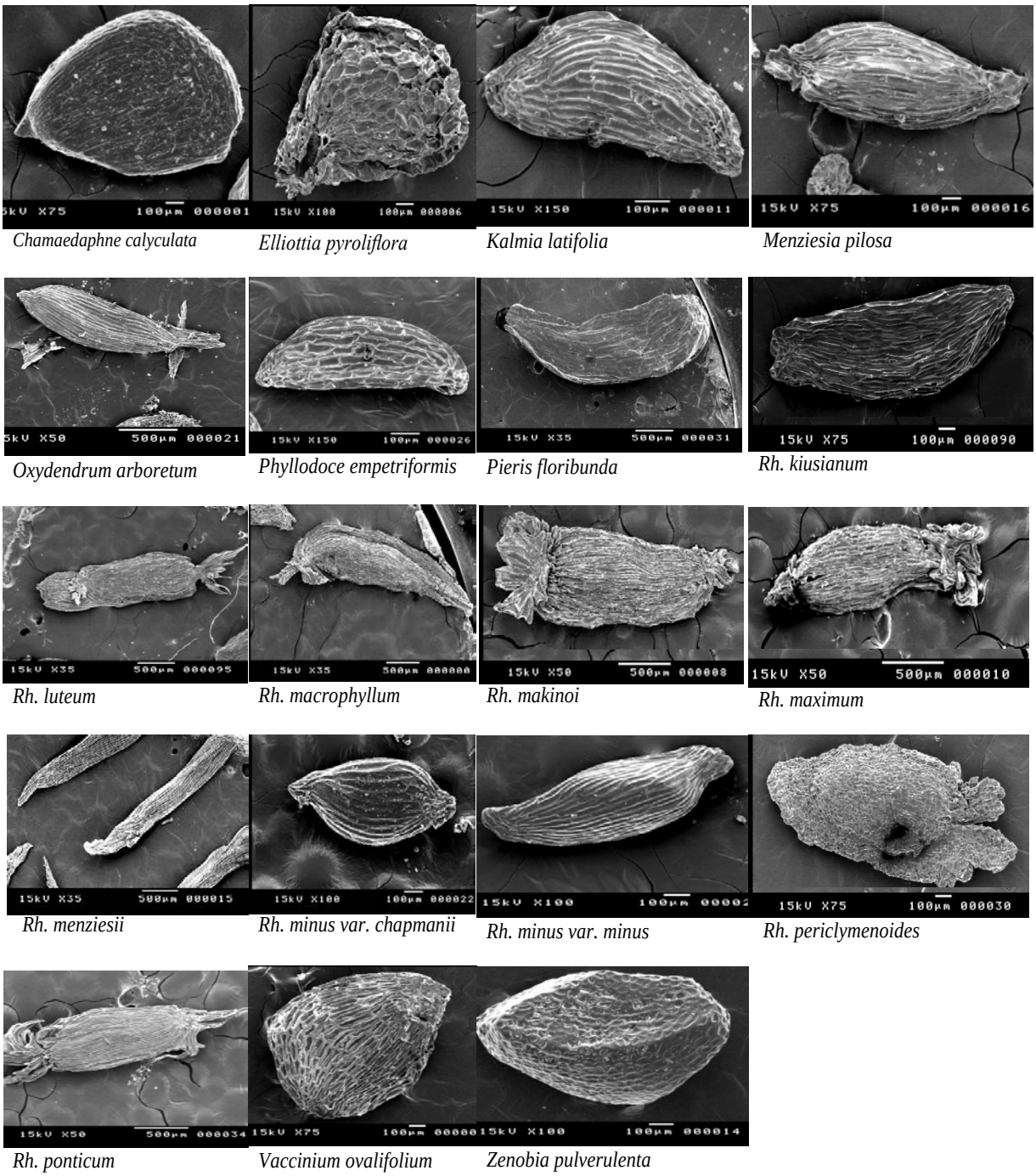

Figure (1): SEM micrographs of 19 mature seeds, showing their macro-morphology

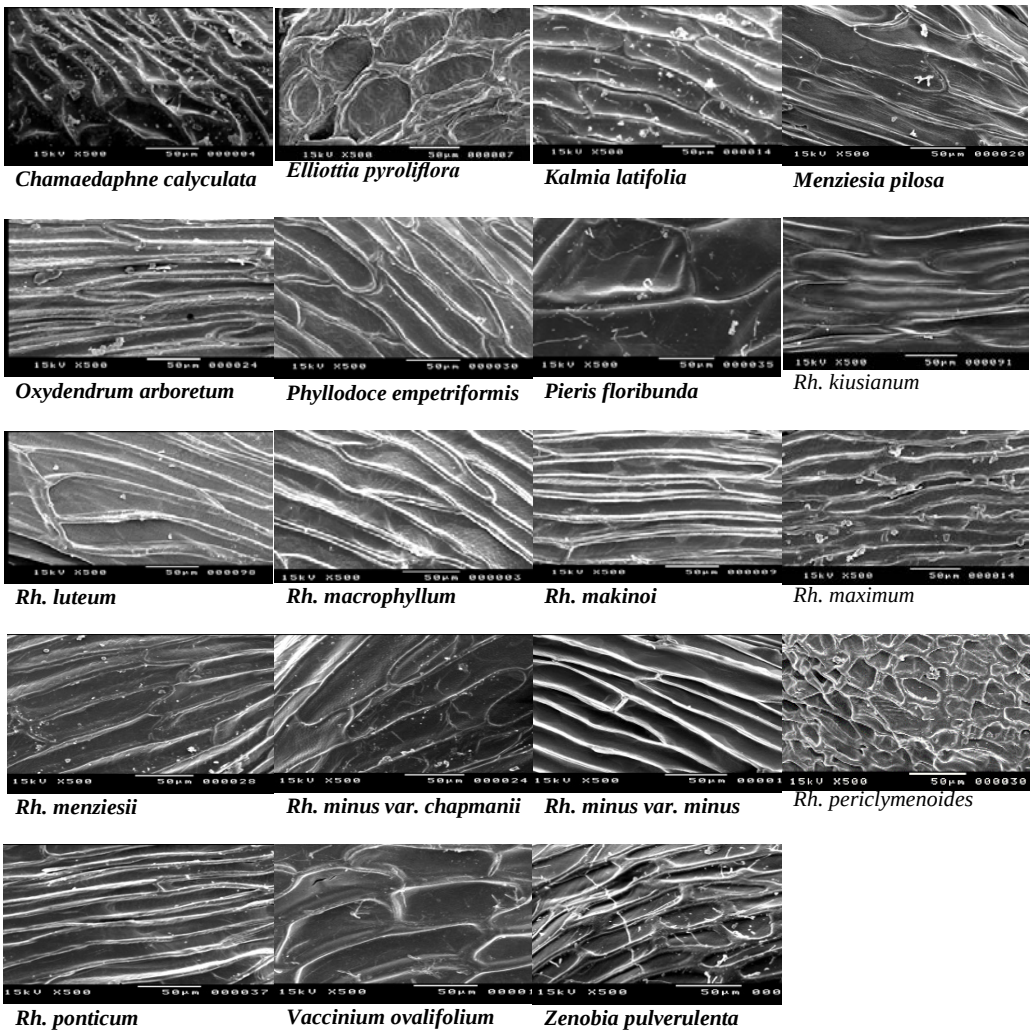

Figure (2): SEM micrographs of 19 mature seeds, showing their pattern of ornamentation

Seed width recoded between 0.51 -1 mm in 10 taxa, while the remaining 9 taxa were recorded with 0.2 - 0.5 mm seed width; consequently, the Length to Width ratio (L/W) was recorded 0.3 - 2.3 in 8 taxa and 2.31 - 3.6 in the 9 taxa, while the highest ratio (3.61 - 8.9) was recorded in only two species: *Oxydendrum arboretum* and *Rhododendron menziesii*. Two seed colors are recorded, the majority of taxa (14 taxa) were recorded with brown seed, while the rest 5 taxa were with yellow seed color.

As illustrated in Figure (2), all seeds are with reticulate pattern, the majority of taxa (15 taxa) has elongated cells with lengths more than or equal to 0.121 mm, the rest four taxa are characterized by relatively short cells with lengths less than or equal to 0.012 mm. The only species *Rhododendron makinoi* reported narrow cells less than or equal to 0.013 mm width, while the majority (18 taxa) reported wider cells more than 0.013 mm width. The cell elongation coefficient was calculated as L/W ratio of the cell, it recorded 0.5 -12 ratio in the majority of specimens (14 taxa), while the rest 5 taxa reported higher ratios (12.1 - 22).

Table (3): The molecular weights (Mw) of protein bands of 19 Ericaceae taxa, numbers 1-19 refer to taxa (Table 1), numbers 28 – 47 refer to protein bands as recorded in combined data matrix 1=present, 0=Absent.

No	RF	Mw	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
28	0.413	**147.043**	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
29	0.45	**122.204**	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
30	0.472	109.472	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
31	0.308	**67.712**	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	0.31	67.252	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
33	0.346	59.484	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
34	0.661	**42.542**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
35	0.67	40.67	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	1	0	0
36	0.465	39.646	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1
37	0.506	34.474	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1
38	0.714	32.637	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0
39	0.826	18.641	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
40	0.753	14.891	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1
41	0.888	13.671	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
42	0.934	10.9	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1	0	0	0	0
43	0.945	**10.28**	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
44	0.881	9.6	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
45	0.964	9.35	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	1	1	0	0
46	0.901	8.967	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
47	0.925	**8.262**	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The pattern of sculpture shows three states of cell shape character i.e., extended polygonal (in 10 taxa); rectangular (in 8 taxa), while only *Elliottia pyroliflora* shows hexagonal cell shape. Anticlinal wall thickness shows two measurement states: ≤ 0.005 mm recorded in 6 taxa; while > 0.005 mm recorded in the remaining 13 taxa.

The seed protein analysis showed a total of 20 bands with various distributions in the 19 taxa. Molecular weights of these bands ranged from 147.04 to 8.262 kDa. Only six bands with molecular weights: 147.043 kDa, 122.204 kDa, 67.712 kDa, 42.542 kDa, 10.28 kDa, 8.262 kDa, are unique in five taxa: *Menziesia pilosa*, *Pieris floribunda*, *Rhododendron makinoi*, *Rhododendron minus* var. *chapmanii* and *Rhododendron ponticum* respectively (Table 3). These bands are considered good specific markers can be used to distinguish these taxa from the remaining. Other bands were polymorphic.

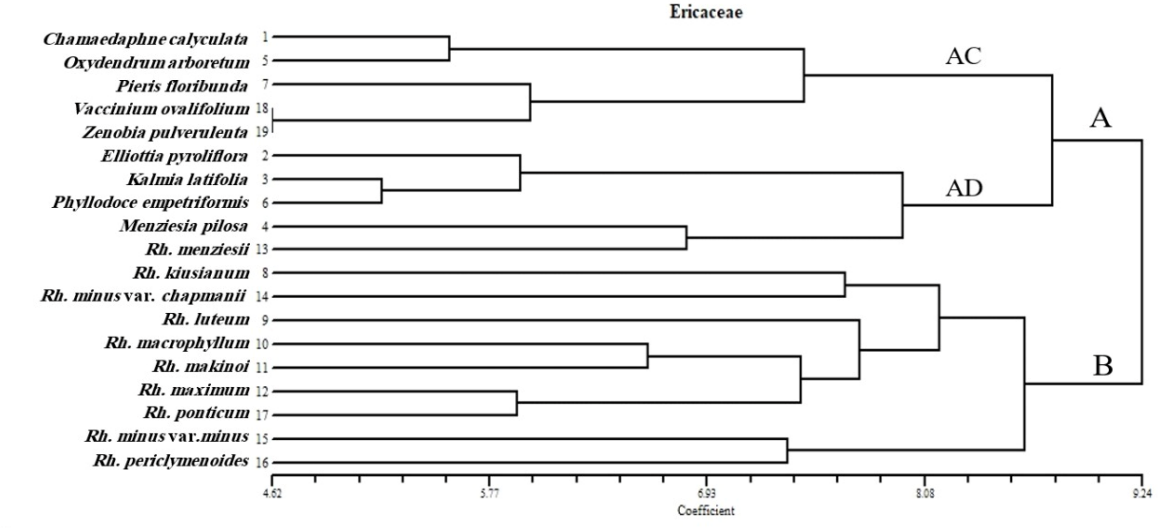

Figure (3): UPGMA dendrogram illustrating the hierarchical phenetic relationships between 19 taxa of Ericaceae based on numerical analysis of seed morphological characters and protein patterns, using a combination of the Sorensen's measures of similarity and Ward's clustering method.

Clustering analysis

The UPGMA dendrogram (Figure 3) showing clustering of the studied taxa based on all 47 characters produced from combination of 27 seed morphological character states and 20 from seed protein patterns. This phenogram revealed that, both major clusters (A) and (B) are separated. The first cluster (A) comprises ten taxa and further divided equally (5 taxa each) into subordinate groups (AC

and AD). The taxa included in the subordinate group (AC) are: *Chamaedaphne calyculata, Oxydendrum arboretum, Pieris floribunda, Vaccinium ovalifolium* and *Zenobia pulverulenta*, this may due to sharing the same seed width category (0.51-1 mm), brown seed color, categories of cell length (≤ 0.12 mm), cell width (> 0.013mm) and anticlinal wall thickness (≤ 0.005 mm). While the taxa included in the subordinate group (AD) are: *Elliottia pyroliflora, Kalmia latifolia, Phyllodoce empetriformis, Menziesia pilosa* and *Rhododendron menziesii* due to sharing the same categories of seed width (0.2-0.5 mm), cell length ≥ 0.121mm), cell width (> 0.013mm) and extended polygonal cell shape. The second major group (B) includes the remaining nine taxa, including all *Rhododendron* taxa.

Discussion

The resulted dendrogram (Figure 3) shows the taxonomic arrangement of the studied 19 Ericaceae taxa in two major groups A and B, the former one was subdivided into subordinate groups AC and AD. The placement of the studied 10 genera were illustrated in different traditional classifications (Drude 1889; Stevens 1971; Takhtajan 1997 and Kron et al. 2002) in comparison with the taxonomic groups of this study (Table 4), which resulted from the combination of seed macro-, micro-morphology and protein pattern (Table 2 & 3). According to the latest classification of Ericaceae (Kron et al. 2002), the family is divided into 8 subfamilies and 20 tribes. The studied genera are distributed equally (5 genera each) in two subfamilies Vaccinoideae and Ericoideae with their different tribes. The taxa clustered in subordinate groups (AC) represents the subfamily Vaccinoideae are distributed in five tribes: *Vaccinium ovalifolium* (Vaccinieae); *Chamaedaphne calyculata* (Gaultherieae); *Oxydendrum arboretum* (Oxydendreae), *Pieris floribunda* (Lyonieae); *Zenobia pulverulenta* (Andromedeae). The taxa clustered in subordinate groups (AD) in addition to those of group (B) represent the subfamily Ericoideae are distributed in two tribes: Phyllodoceae (*Elliottia pyroliflora, Kalmia latifolia* and *Phyllodoce empetriformis*); and tribe Rhodoreae (*Menziesia pilosa* and all *Rhododendron* taxa).

This hierarchical taxonomic arrangement typically matches the classifications of Drude (1889); Stevens (1971); Takhtajan (1997) and Kron et al. (2002) as illustrated in Table (4).

As illustrated above, the results of this preliminary cladistics analysis supports a wider classification of Ericaceae. Due to the limited number of studied taxa, our analyses are not extensive enough to address detailed overview of subfamilial and tribal relationships within the family. But at least it matched the arrangement of the taxa into the two distinct subfamilies Vaccinoideae and Ericoideae. Few points, however, are clear such as the close relationship among *Chamaedaphne calyculata* and *Oxydendrum arboretum* which reported in the previous classifications (Drude 1889; Stevens 1971 and Takhtajan 1997) that placed them in one tribe (Andromedeae) is confirmed here by clustering them in one subgroup although belonging recently to different two tribes Gaultherieae and Oxydendreae respectively according to Kron et al. (2002), Table (4).

Table (4): The placement of the studied genera in different classification systems. Subfamilies are in boldface; tribes are between brackets.

Genera	Drude 1889	Stevens 1971	Takhtajan 1997	Kron et al. 2002	Fig. 1
Vaccinium	**Vaccinoideae*** (Vaccinieae)	**Vaccinoideae** (Vaccinieae)	**Vaccinoideae** (Vaccinieae)	**Vaccinoideae** (Vaccinieae)	
Chamaedaphne	**Arbutoideae** (Andromedeae)	(Andromedeae)	(Andromedeae)	(Gaultherieae)	
Oxydendrum	(Andromedeae)	(Andromedeae)	(Andromedeae)	(Oxydendreae)	**AC**
Pieris	(Andromedeae)	(Andromedeae)	(Andromedeae)	(Lyonieae)	
Zenobia	(Andromedeae)	(Andromedeae)	(Andromedeae)	(Andromedeae)	
Elliottia	**Rhododendroideae** (Ledeae)	**Rhododendroideae** (Cladothamneae)	**Rhododendroideae** (Cladothamneae)	**Ericoideae** (Phyllodoceae)	
Kalmia	(Phyllodoceae)	(Phyllodoceae)	(Phyllodoceae)	(Phyllodoceae)	**AD**
Phyllodoce	(Phyllodoceae)	(Phyllodoceae)	(Phyllodoceae)	(Phyllodoceae)	
Menziesia	(Rhodoreae)	(Rhodoreae)	(Rhododendreae)	(Rhodoreae)	
Rhododendron	(Rhodoreae)	(Rhodoreae)	(Rhododendreae)	(Rhodoreae)	**B**

*recognized as family level by Hooker (1876) and Hutchinson (1973)

The three species: *Pieris floribunda*, *Vaccinium ovalifolium* and *Zenobia pulverulenta* appeared closely related in one subgroup because of sharing the, brown seeds with rounded ends, and rectangular cell shape, also having the same categories of seed width (0.51 -1mm), cell length (≤ 0.12 mm), cell width (> 0.013 mm) and anticlinal wall thickness (≤ 0.005 mm), in addition to recording three protein bands with molecular weights 39.646 kDa, 34.474 kDa and 14.891 kDa (Table 3), this clustering reflects belonging to the subfamily Vaccinoideae, although the clustering of *Vaccinium ovalifolium* and *Zenobia pulverulenta* separately due to sharing 13 characters (see Appendix), this is not in accordance with the cladistics classifications of Drude (1889); Stevens (1971) and Takhtajan (1997). According to Bidartondo & Bruns (2001) based on molecular phylogenetic, they observed a close affinity between *Vaccinium* sp. and *Oxydendrum arboretum*, that is differ slightly from our result. According to Kron & Creel (1999) based on matK sequence data, they grouped *Vaccinium* sp. in Vacciniids group and put *Pieris* sp. in Lyonia group, that is differ from our result which grouped *Pieris floribunda* and *Vaccinium ovalifolium* in a small subgroup (AC).

The precise placement of *Vaccinium ovalifolium* in very near distance with the other members of tribe Andromedeae *sensu* Drude (1889); Stevens (1971) and Takhtajan (1997) is problematic, because of its position in a distinctive tribe Vaccinieae or even in a separate family by Hooker (1876) and Hutchinson (1973). Actually, it isn't closely related to *Pieris floribunda* and *Zenobia pulverulenta* according to Kron et al. (2002), but here due to the reduced number of tribe representatives, the clustering may be considered at a generic level. Vaccinioideae is a very heterogeneous subfamily, with the highest number of genera. It consists of 5 tribes, comprising 45 genera and about 1600 species (Kron & Luteyn, 2005). The genera included in this subfamily were previously the members of tribes Andromedeae and Vaccinieae *sensu* Drude (1889); Stevens (1971) and Takhtajan (1997).

The five taxa which are clustered in the small subgroup (AD) at the similarity level 7.97 split off into two levels. Three taxa (*Elliottia pyroliflora, Kalmia latifolia* and *Phyllodoce empetriformis*), represented the tribe Phyllodoceae according to Kron et al. (2002), are grouped separately due to sharing 7 seed characters (see Appendix) and three polymorphic protein bands with molecular weights: 39.646 kDa, 34.474 kDa and 14.891 kDa (Table 3).

Furthermore, *Elliottia pyroliflora* splits off at a separate line because having ovate seed, hexagonal cell shape and the monomorphic protein band with the molecular weight 67.712 kDa, which matches the placement under tribe Ledeae by the classifications of Drude (1889); and under tribe Cladothamneae by Stevens (1971) and Takhtajan (1997). At the same time, the clustering of *Kalmia latifolia* and *Phyllodoce empetriformis*, may due to having the oblong seeds and rectangular cell shape, confirmed the close relationship and the placement under tribe Phyllodoceae by the all above mentioned classification systems (Table 4).

Menziesia pilosa and *Rhododendron menziesii* are grouped at the similarity level of 6.81 because of sharing some characters as the rounded seed end and the same categories of seed length (1.41 -2.6 mm), L/W ratio (3.61 - 8.9), anticlinal wall thickness (> 0.005 mm). The clustering of *Menziesia pilosa* with *Rhododendron menziesii* together and their arrangement in a very near distance of the remaining *Rhododendron* taxa (main group B) (Figure 3) confirmed the placement of the genera *Menziesia* and *Rhododendron* under tribe Rhodoreae *sensu* Drude (1889); Stevens (1971) and Kron et al. (2002), or under tribe Rhododendreae by Takhtajan (1997). Craven (2011) suggested the transfer of the genus *Menziesia* to be nested in *Rhododendron* based upon cladistics analyses of DNA data. That result is confirmed here by sharing *Menziesia pilosa* of 12 micro-, macro- seed morphological characters and the protein pattern with one or more *Rhododendron* taxa (Appendix).

The morphological variations between *Menziesia* and *Rhododendron* are not so great (Craven, 2011), for example: the anther dehiscence by slits in both genera. Viscin threads arise among the pollen grains in *Rhododedndron* playing a role in pollen dispersal from anthers and its adhesion to pollinators. In contrast, Stevens et al. (2004) and Kita et al. (2005) reported the lack of viscin threads in *Menziesia,* while Copeland (1943) reports viscin threads in *Menziesia*. Stevens et al. (2004) and Fang et al. (2005) also differentiate *Menziesia* from *Rhododendron* by capsule shape i.e. subspheroidal in *Menziesia,* while longer than wide in *Rhododendron.* The morphological evidence by Copeland (1943), Stevens et al. (2004) and Fang et al. (2005), in addition to the molecular data by Craven (2011) supported the inclusion of *Menziesia* in *Rhododendron.* Although, this result is confirmed in the present study using seed characters and protein pattern, this will require testing a greater number of species from both genera with a strong sampling of species, and with datasets preferably the molecular ones. Especially, there were different experimental reports (Handa et al. 2003; 2006; Kita et al. 2005) recorded the generic hybridization between *Menziesia* and *Rhododendron*, so that, the real relationship between both genera needs more studies.

The remaining nine *Rhododendron* taxa are clustered in a separate clade (B) (Figure 3), due to sharing the majority of seed and protein pattern characters (Appendix), this arrangement confirmed their placement in the subfamily Rhododendroideae *sensu* Drude (1889); Stevens (1971) and Takhtajan

(1997), or recently under Ericoideae *sensu* Kron et al. (2002).

The infra-generic relationships within genus *Rhododendron* had been discussed previously by Shalabi et al. (2020) based on the data of seed morphology and protein analyses of 28 *Rhododendron* taxa.

This study is considered a complementary one to Shalabi et al. (2020) to find and confirm the taxonomic relationships between some Ericaceae taxa, using the combination of seed macro/ micro – morphology and SDS-Page protein pattern. Due to the limited number of studied taxa, our analyses are not extensive enough to address detailed overview of subfamilial and tribal relationships within the family. The new results which were obtained in this study will be useful in updating / confirming the taxonomic relationships within the Ericaceae, in the case of incorporating more taxa. More taxonomic studies based on the molecular data are recommended using consistent characters, and taxa in a wide range of classificatory levels.

Acknowledgments

The authors are thankful to the botanical gardens, herbaria and arboreta of the University of British Columbia (UBC), Canada, The Dawes Arboretum (DAWES), United States of America, and Polish Academy of Sciences (PAN) for providing the required seeds.

References

Barclay-Estrup, P. & Gimingham, C.H. 1994. Seed-shedding in a Scottish heath community. *Journal of Vegetation Science*, 5: 197–204.

Barthlott, W. & Hunt, D.R. 2000. Seed-diversity in the Cactaceae subfam. Cactoideae. *Succulent Plant Research*, 4: 1–173.

Barthlott, W. & Voit, G. 1979. Mikromorphologie der Samenschalenund Taxonomie der Cactaceae: ein raster-elektronenmikroskopischerUberblick. – *Pl. Syst. Evol*., 132: 205-229.

Bidartondo, M.I. & Bruns T.D. 2001. Extreme specificity in epiparasitic Monotropoideae(Ericaceae): widespread phylogenetic andgeographical structure. *Molecular Ecology*, 10: 2285–2295.

Chamberlain, D., Hyam, R., Argent, G., Fairweather, G. & Walter, K.S. 1996. *The Genus Rhododendron: its Classification and Synonymy*. Royal Botanic Garden, Edinburgh.

Christenhusz, M. J. M. & Byng, J. W. 2016. The number of known plants species in the world and its annual increase. *Phytotaxa*, 261(3): 201–217.

Copeland, H.F. 1943. A study, anatomical and taxonomic, of the genera of Rhododendroideae. *American Midland Naturalist*, 30: 533– 625.

Craven, L. A. 2011. *Diplarche* and *Menziesia* transferred to *Rhododendron* (Ericaceae). *Blumea*, 56: 33–35.

Craven, L.A., Goetsch, L.A., Hall, B.D. & Brown, G.K. 2008. Classification of the *Vireya* group of *Rhododendron* (Ericaceae). *Blumea*,53:435-42.

Drude, O. 1889. Ericaceae. Pp. 15– 65 in Die Natu¨ rlichen Pflanzenfamilien vol. 4(1), eds. Engler A. and K. Prantl. Leipzig: W. Engelmann.

Fagundez, J. & Izco, J. 2003a. Seed morphology of *Erica* L. Sect. Callicodon Bentham. *Plant biosystems*, 137: 111-116.

Fagundez, J. & Izco, J. 2003b. Seed morphology of *Erica* L. Sect. Chlorocodon Bentham. *Acta Bot. Gallica*, 150(4): 401-410.

Fagundez, J. & Izco, J. 2004. Seed morphology of *Calluna* Salisb. (Ericaceae). *Acta Botanica Malacitana*,29: 215-220.

Fagundez, J. & Izco, J. 2011. Seed morphology and anatomy of the Mediterranean pentamerous species of *Erica* (Ericaceae). *Turk J Bot*., 35: 643-651.

Fang, M.Y., Fang, R.Z, He, M.Y., Hu, L.C., Yang, H.P., Qin, H.N., Ming, T.L., Chamberlain, D.F., Stevens, P.F., Wallace, G.D. & Anderberg A. 2005. Ericaceae. – In: Wu, Z., Raven, P.H., *Flora of China*, 14: 242–517.

Fraga, M.I. 1984. Valor taxonómico de lamorfología de las semillas en las especies delgenero *Erica* presentes en el NO de España. *Acta Bot. Malacitana*, 9: 147-152.

Fredes M., Muñoz C., Prat L., Torres F., Sáez P., Bustamante L., Paiva L., & Pertuzé R. 2016. Seed morphology and anatomy of *Rubus geoides* Sm. *Chilean Journal of Agricultural Research*,76(3): 385-389

Granström, A.1987. Seed viability offourteen species during five years of storagein a forest *soil. J. of Ecol*., 75: 321-331.

Handa, T., Kita, K., Wongsawad, P., Kurashige, Y. & Yukawa, T. 2003. Molecular phylogeny as a guide for breeding of ornamentals: the case study of *Menziesia* and *Dendrobium*. *Acta Horticulturae*, 612: 155–163.

Handa, T., Kita, K., Wongsawad, P., Kurashige, Y. & Yukawa, T. 2006. Molecular phylogeny-assisted breeding of ornamentals. *Journal of Crop Improvement*, 17: 51–68.

Heads, M., 2003. Ericaceae in Malesia: vicariance biogeography, terrane tectonics and ecology.*Telopea*, 10(1): 311–449

Hooker, J.D. 1876. Ericaceae. In: Bentham G, Hooker JD, eds. *Genera Plantarum*, Vol. 2. London: Reeve.

Hutchinson, J. 1973. *The families of flowering plants*. Oxford University Press, London.

Kita, K., Kurashige, Y., Yukawa, T., Nishimura, S. & Handa, T. 2005. Intergeneric hybridization between *Menziesia* and *Rhododendron* based on molecular phylogenetic data. *Journal of the*

Japanese Society for Horticultural Science, 74: 51–56.

Kron, K.A. & Creel, M. 1999. new species of deciduous *Azalea* (*Rhododendron* section Pentanthera; Ericaceae) from South Carolina. *Novon: A Journal of Botanical Nomenclature*, 9(3):377-380.

Kron, K.A. & Luteyn, J.L. 2005. Origins and biogeographic patterns in Ericaceae: New insights from recent phylogenetic analyses, *Biol. Skr*. 55:479–500.

Kron, K.A., Judd, W.S., Stevens, P.F., Crayn, D.M. Anderberg, A.A., Gadek, P.A., Quinn, C.J. & Luteyn, J.L. 2002. Phylogenetic classification of Ericaceae: Molecular and morphological evidence. *The Bot. Review*, 68(3): 335-423.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of head bacteriophage T4. *Nature*, 227: 680-685.

Olson, A.R. 1980.Seed morphology of *Monotropa uniflora* L. (Ericaceae). *Amer. J.of Bot*., 67(6): 968-974.

Pons, T.L. 1989. Dormancy and germination of *Calluna vulgaris* (L.) Hull and *Erica tetralix* L. seeds. *Acta Oecol. Oecol. Plant*, 10: 35–43.

Rohlf, F. J., & Sokal, R.R., 1981. Comparing Numerical Taxonomic Studies. *Syst. Zool.,* 30(4): 459-490.

Rohlf, F.J. 2005. NTSYS-pc, *Numerical taxonomy and multivariate analysis system*. New York: Exeter Computers.

Shalabi, L.F., Hamdy, N. & Hussein, E.A. 2020. Numerical classification of *Rhododendron* (Ericaceae) based on seed morphology and protein profile. *Phytologia Balcanica*, 26 (3): 471 – 478.

Stevens, P.F. 1971. A classification of the Ericaceae: Subfamilies and tribes. *J. Linn. Soc., Bot.* 64: 1-53.

Stevens, P.F., Luteyn, J., Oliver, E.G.H., Bell, T.L., Brown, E.A., Crowden, R.K., George, A.S., Jordan, G.J., Ladd, P., Lemson, K., McLean, C.B., Menadue, Y., Pate, J.S., Stace, H.M. & Weiller, C.M. 2004. Ericaceae. In: Kubitzki K, *The families and genera of vascular plants* 6: 145–194. Springer, Berlin & Heidelberg.

Takahashi, H. 1993. Seed morphology andits systematic implications in Pyroloideae (Ericaceae). *Int. J. of Plant Sci.,* 154(1): 175-186.

Takhtajan, A.L. 1997. *Diversity and classification of flowering plants.* Columbia Univ. Press, New York.

Vander, K.S.P. 1983. Seed and seedling characters in *Vaccinium* gr. *myrtillus*. *– LeNat. Canadien*, 110: 285-292.

Appendix: Combined data matrix including 47 characters recorded for 19 Ericaeae taxa, numbers from 1-19 refer to taxa (see Table 1), numbers from 1 -27 refer to seed morphological characters (see character list Table 2), numbers from 28 -47 refer to the protein bands with 20 molecular weights (see Table 3), 1=present, 0=absent.

taxa	Seed macro- / micro- morphological characters																										Protein bands with 20 molecular weights																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
1	0	0	0	1	0	0	1	1	0	0	1	1	0	0	1	0	1	0	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
2	0	0	0	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0
3	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0
4	0	0	1	0	0	1	0	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0
5	0	0	1	0	0	1	0	1	0	0	1	1	0	0	1	0	1	0	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
6	0	1	0	0	0	0	1	1	0	1	0	0	1	0	0	1	0	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0
7	0	1	0	0	0	0	1	0	1	0	1	0	1	0	1	0	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0	0	1	0	0	0
8	0	0	1	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
9	0	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1	0	1	1	1	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0	0
10	0	0	0	1	0	1	0	0	1	0	1	0	1	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	1	0	0	1	0	0
11	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0	1	1	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
12	0	0	1	0	0	1	0	0	1	1	0	0	1	0	0	1	0	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
13	1	0	0	0	0	1	0	0	1	1	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
14	0	1	0	0	0	1	0	1	0	1	0	1	0	0	1	0	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
15	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0
16	0	0	0	0	1	0	1	1	0	0	1	0	1	0	1	0	0	1	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0
17	0	1	0	0	0	1	0	0	1	1	0	0	1	0	0	1	0	1	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
18	0	0	0	1	0	0	1	1	0	0	1	1	0	0	1	0	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0	0	1	0	0	0
19	0	0	0	1	0	0	1	1	0	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0